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Dependence of Ca^{2+} and K^+ Current Development on RNA and Protein Synthesis in Muscle-Lineage Cells of the Ascidian *Boltenia villosa*

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The early development of excitability of muscle-lineage cells of the ascidian *Boltenia villosa* is characterized by the appearance, just after gastrulation, of a Ca^{2+} current and a delayed outward K^+ current, while an inwardly rectifying K^+ current, present since fertilization, disappears. The muscle-lineage cells are the first cells in which we detect tissue-specific electrical properties after gastrulation. Here, we show that the development of electrical properties in these cells involves RNA and protein synthesis. If transcription or translation is blocked, the Ca^{2+} and outward K^+ currents fail to appear, whereas the inward K^+ current disappears normally. For the Ca^{2+} current, the sensitive period for transcription extends until just before gastrulation, while the sensitive period for translation extends until after gastrulation. The oocyte has a Ca^{2+} current present at about 5–10% the density of that in the muscle-lineage cells; this current disappears by gastrulation. A comparison of the oocyte and muscle Ca^{2+} currents indicates that they are similar in voltage dependence and inactivation mechanism. A small difference in permeability sequence can be attributed to different surface charge properties at the two stages of development.

Ion channels are among the proteins that become expressed in a tissue-specific manner during embryogenesis. The resulting differences in functional ion channel populations among mature cells underlie the diversity of their electrical properties. Because both agonist-gated and voltage-dependent ion channels are present in most oocytes before fertilization, the electrical differentiation of cells in the embryo involves the modification of oocyte channel populations as well as the cell-specific appearance of new channel types. It has been shown in several cases that the expression of mature channel populations during terminal differentiation of nerve and muscle depends on RNA synthesis (O'Dowd, 1983; Hirano and Takahashi, 1987), and in other experiments, the appearance of mRNA for specific ion channels has been detected directly (Cooperman et al., 1987; Offord and Catterall, 1989; Scheinman et al., 1989). In amphibian spinal neurons, the transcription must occur during a certain critical period if delayed K^+ current is to appear, implying the transient presence at that time of an activator of transcription (Ribera and Spitzer, 1989).

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Some proteins that show stage-specific expression during development depend on *de novo* RNA synthesis; others do not. In the ascidian *Ciona,* AChE, an enzyme specific to muscle cells, does not develop if RNA synthesis is blocked before gastrulation (Meedel and Whittaker, 1983), whereas alkaline phosphatase, an enzyme specific for the endodermal tissue, develops normally even after RNA synthesis block (Whittaker, 1977). Thus, the appearance of AChE after gastrulation depends on RNA that is first synthesized just prior to that stage, whereas alkaline phosphatase RNA appears to be localized in the egg cytoplasm, segregated during early cleavage into the endodermal cell lineage of the embryo and later translated.

We have studied the tissue-specific appearance of voltage-gated ion channels during early development of the ascidian *Boltenia villosa.* In *Boltenia,* the unfertilized egg has Na^+, Ca^{2+}, and inwardly rectifying K^+ currents (Block and Moody, 1987; Hice and Moody, 1988). Between fertilization and the gastrula stage, the Na^+ and Ca^{2+} currents disappear from all cells, while the inwardly rectifying K^+ current is maintained at approximately constant density (Block and Moody, 1987; Simoncini et al., 1988). By the neurula stage, 3–5 hr after gastrulation, a Ca^{2+} current is again expressed in muscle-lineage cells, but not in cells of other lineages, at more than 10 times the density found in the oocyte; muscle-lineage cells also preferentially develop a delayed outward K^+ current during this time, while cells of all lineages lose the inwardly rectifying K^+ current (Simoncini et al., 1988). Thus, the muscle cells are the first to develop tissue-specific ion channels.

The extremely rapid time course of this electrophysiological differentiation and the fact that nonmuscle cells in the embryo undergo only a subset of the changes seen in the muscle-lineage cells make this an ideal preparation in which to study the mechanisms underlying the cell-specific expression of ion channel populations. We have studied the development of electrical properties of these muscle cells to determine whether RNA and protein synthesis are necessary for these changes. We found that the development of Ca^{2+} currents and delayed outward K^+ currents in the embryo is sensitive to blockade of RNA and protein synthesis near the time of gastrulation, while the disappearance of the inward rectifying K^+ current is not.

Materials and Methods

Preparation of gametes. Specimens of the ascidian *Boltenia villosa* were collected from the Puget Sound, Washington, and maintained in holding tanks in the Sound. For fertilization, oocyte and sperm were obtained from pairs of animals as follows: gonads were removed from the animals, minced in iced seawater, and rapidly washed with several hundred milliliters of 10–12°C seawater, sequentially through 223-μm and 102-

μm Nitex filters. The first filter retains gonadal debris, and the second retains oocytes but passes sperm. Sperm were collected as the first wash through the 102-μm filter. Oocytes were allowed to sit for 1 hr in cold seawater and then fertilized with heterologous sperm. Eggs were dechorionated either manually with electrolytically sharpened tungsten needles or enzymatically (Mita-Myazawa and Satoh, 1986). Cells from later embryos were dissociated by one of two methods: (1) by incubation for 5–15 min in a low-Ca^{2+} collagenase medium containing 460 mM NaCl, 10 mM KCl, 0.5 mM $CaCl_2$, 10 mM HEPES (pH, 7.5), and collagenase (1 mg/ml; Sigma type IV), followed by trituration in artificial seawater (ASW); or (2) by 15 min incubation in Ca-Mg-free ASW containing 460 mM NaCl, 10 mM KCl, 10 mM HEPES (pH, 8), followed by vortexing (modified from Mita-Myazawa and Satoh, 1986). Muscle cells were recognized by the presence of an intense orange pigment that enables them to be identified at any stage of development, even in dissociated preparations (Simoncini et al., 1988). We are unable as yet to distinguish among the various nonmuscle (unpigmented) lineages.

External solutions. ASW had the following composition (in mM): 400 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, and 10 HEPES; pH was adjusted to 8 with NaOH. Ba-ASW, Sr-ASW, and Ca-ASW contained (in mM) 10 $MgCl_2$ and 50 $BaCl_2$, 50 $SrCl_2$, or 50 $CaCl_2$, respectively. Actinomycin D (Sigma) was used at 20 μg/ml in ASW (Whittaker, 1977). Emetine (Sigma) was used at 100 μM in ASW. Drugs were removed just prior to recording so that currents were measured in control solutions. When either actinomycin D or emetine was applied before gastrulation, the normal movements associated with gastrulation were disrupted. The disruption of morphogenetic movements and cell interactions is unlikely by itself to explain the effects of these drugs on development of ion channel properties, because cells dissociated from the embryo at gastrulation undergo normal electrophysiological development (Simoncini et al., 1988).

Internal solutions. K-int solution contained (in mM) 200 or 400 KCl, 10 NaCl, 1 $MgCl_2$, 10 EGTA, 20 HEPES, 2 theophylline, 0.1 cAMP, and 2 ATP; pH, 7.3. Cs-int substituted 400 CsCl for 400 KCl.

Electrical recording. All recordings were done with the whole-cell variation of the patch-clamp technique (Hamill et al., 1981). Electrophysiological methods and data acquisition were as described previously (Block and Moody, 1987; Simoncini et al., 1988). Current density was measured by dividing total cell current by cell capacitance. Cell capacitance was measured by applying a 10-mV, 50-Hz triangle waveform to the command input. The current response to a triangle wave voltage command is a square wave whose amplitude is proportional to capacitance. Total capacitance was calculated as $C_M = I/(2\ dV/dT)$, where I is the amplitude of the square step in current signal produced as the slope of the triangle wave command voltage changes from $+(dV/dt)$ to $-(dV/dt)$ [i.e., $\Delta(dV/dt) = 2(dV/dt)$]. Because the measurement is made at a point where there is an instantaneous change in dV/dt, but not V itself, this method is independent of membrane conductance.

Results

We previously analyzed the development of voltage-dependent ion currents in muscle-lineage cells using the whole-cell variation of the patch-clamp technique (Hamill et al., 1981). A summary of the normal progression of electrical properties in muscle cells of *Boltenia villosa* is shown in Figure 1 (Block and Moody, 1987; Simoncini et al., 1988). Four principal ion currents were examined: (1) An inwardly rectifying K^+ current is present in all cells from before fertilization through gastrula stage at densities between −1.5 and −7.8 pA/pF (measured at −130 mV in Sr-ASW). The density of the inward rectifier increases slightly between fertilization and gastrulation, indicating substantial active regulation because total membrane area increases about 10-fold during this time. The inward rectifier decreases abruptly after gastrulation and is absent in all muscle cells (and in all other cells sampled) by early tailbud stage. (2) A delayed K^+ current, which is not present in any cell before tailbud stage, appears primarily, but not exclusively, in muscle cells between gastrulation and the early tailbud stage. (3) An inward Ca^{2+} current is present in the egg before fertilization and is maintained at constant density in all blastomeres through the 8-cell stage, despite a threefold increase in the total surface area of the embryo. By gastrula stage (116–176 cells), the Ca^{2+} current is not detectable in any cell studied (stages between 8-cell and gastrula have not been examined). By early tailbud stage, the Ca^{2+} current reappears exclusively in muscle-lineage cells at densities about 20-fold greater than in the oocyte (range, −2.6 to −47.2 pA/pF; average, −13.4 pA/pF; $n = 8$). (4) Not shown in Figure 1 is the developmental profile of the Na^+ current. This current is present in the oocyte, but disappears within 2 hr after fertilization, at the time of first cleavage. We have not detected the reappearance of Na^+ currents in any cells at later stages through neurulation.

Effects of RNA and protein synthesis blocker on the Ca^{2+} current

The presence of a Ca^{2+} current in the egg and the appearance of a similar Ca^{2+} current in muscle-lineage cells after gastrulation pose the question of whether *de novo* synthesis of Ca^{2+} channel mRNA occurs specifically in muscle-lineage cells near the time of gastrulation. Alternatively, preformed maternal mRNA responsible for the expression of Ca^{2+} channels could be partitioned into muscle-lineage cells during early cleavages (Whittaker, 1977). To investigate this question, we treated embryos at different developmental stages with actinomycin D, a drug that inhibits RNA transcription; actinomycin D was then maintained in the culture dish until the embryos reached tailbud stage. Under normal developmental conditions, muscle cells at tailbud stage would have developed Ca^{2+} currents. At this stage, Ba^{2+} (Ba-ASW) currents through Ca^{2+} channels were measured; Ba^{2+} currents are more easily measured than Ca^{2+} currents because they are larger and do not inactivate. The pipette solution contained Cs^+ (Cs-int) to block outward currents. Figure 2*A* shows that Ca^{2+} channels failed to develop when RNA synthesis was blocked before the 44-cell stage, whereas they developed to about −2 to −4 pA/pF of current when RNA synthesis was blocked at gastrula stage. The average Ca^{2+} current density at tailbud stage in normally developing embryos, without drug treatment, was −13.4 pA/pF (n = 8). Figure 2, *B* and *C*, shows current records from muscle cells of tailbud-stage embryos that had been treated with actinomycin D starting at gastrula (*B*) or 32-cell stage (*C*). These results indicate that the RNA responsible for Ca^{2+} channel development is first synthesized near the time of gastrulation and is not present at earlier stages.

The above results imply that the appearance of Ca^{2+} currents should also be sensitive to protein synthesis inhibition, but with a later sensitive period. Figure 3 shows this to be the case. Emetine, a protein synthesis inhibitor (Hogan and Gross, 1971), was applied at different developmental stages, and the currents were measured at tail-bud stage as in the actinomycin D experiments. The sensitive period for protein synthesis inhibition was found to extend through gastrulation (Fig. 3*A*), later than the sensitive period for RNA synthesis. Figure 3 also shows currents recorded from embryos treated with the drug at the neurula stage (Fig. 3*B*) and the 44-cell stage (*C*). These results indicate that synthesis of protein from the mRNA responsible for Ca^{2+} current development follows RNA synthesis by only a few hours.

Effects of RNA and protein synthesis blockade on delayed K^+ current

Delayed outward K^+ current appears predominantly in muscle-lineage cells after gastrulation, at the same time as the Ca^{2+}

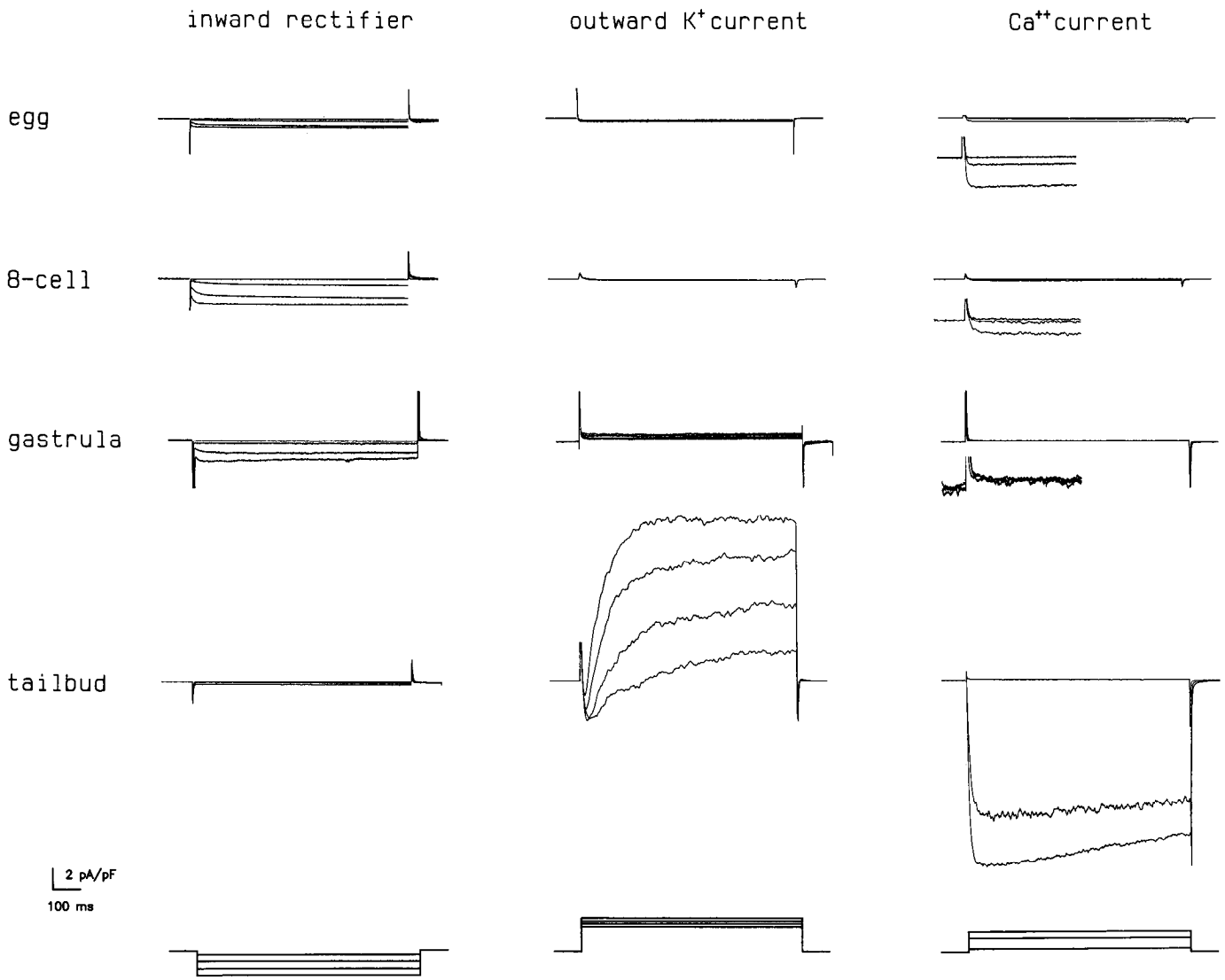

Figure 1. Development of Ca^{2+} and K^+ currents in muscle-lineage cells. The vertical display gain for each set of records has been normalized to the cell capacitance, so that a single vertical current *density* calibration applies to the entire figure. *Left column,* Inwardly rectifying K^+ currents evoked from $V_h = -50$ mV by voltage steps to -60, -80, -100, and -120 mV. External solution was Sr-ASW, and the pipette solution was 200 mM K-int. The capacitances of the cells were as follows: *egg,* 500 pF; *8-cell,* 170 pF; *gastrula,* 40 pF; *tailbud,* 25 pF. *Middle column,* Delayed outward K^+ currents evoked from $V_h = -60$ mV by voltage steps to $+10$, $+20$, $+30$, and $+40$ mV. External solution was Sr-ASW, and the pipette solution was 200 mM K-int. The capacitances of the cells were as follows: *egg,* 500 pF; *8-cell,* 170 pF; *gastrula,* 40 pF; *tailbud,* 25 pF. *Right column,* Ca^{2+} channel currents evoked from $V_h = -60$ mV by voltage steps to -40, -10, and $+10$ mV. The *insets* show currents amplified by a factor of 10. External solution was Ba-ASW, and the pipette solution was Cs-int. The capacitances of the cells were as follows: *egg,* 480 pF; *8-cell,* 150 pF; *gastrula,* 20 pF; *tailbud,* 26 pF. At gastrula stage, the Ca^{2+} current is undetectable. If the Ca^{2+} current had remained at the average density of the 8-cell stage (-0.65 pA/pF in Sr^{2+}; Block and Moody, 1987; -0.93 pA/pF in Ba^{2+}, corrected for the Ba:Sr permeability ratio), we would have expected a mean current of about 30 pA based on a capacitance at gastrula stage of 30 ± 13 pF ($n = 20$). Because our limit of detection is 3–5-pA currents, the Ca^{2+} current is smaller by at least a factor of five at gastrula than at 8-cell stage. Note that the *scale bar* for the *inset* for the gastrula Ca^{2+} current is 0.2 pA/pF or 4 pA absolute.

current, but unlike the Ca^{2+} current, it is not seen at earlier stages (Figs. 1, 4*A*). We did similar blocker experiments on the delayed K^+ current, though we did not attempt to measure the ends of the sensitive periods. Outward K^+ currents (measured at tailbud stage) failed to develop when RNA synthesis was blocked at 32- and 44-cell stages, showing that new (not maternal) RNA is responsible for the appearance of this current (Fig. 4*B*). Appearance of the delayed outward K^+ current was similarly sensitive to blockade of protein synthesis. When emetine was applied between 32-cell and gastrula stages, no current developed ($n = 12$; data not shown).

Effects of RNA and protein synthesis blockade on the inwardly rectifying K^+ current

The inwardly rectifying K^+ current, activated at potentials negative to rest, is present in all cells until gastrulation, but it is greatly reduced at later stages (Fig. 1; Simoncini et al., 1988). Actinomycin D experiments were done to test whether the disappearance of this current depended on RNA synthesis. Figure 5*A* shows that, when actinomycin D was applied at the 32-cell stage and maintained in the culture dish, the inwardly rectifying K^+ current disappeared normally (see Simoncini et al., 1988,

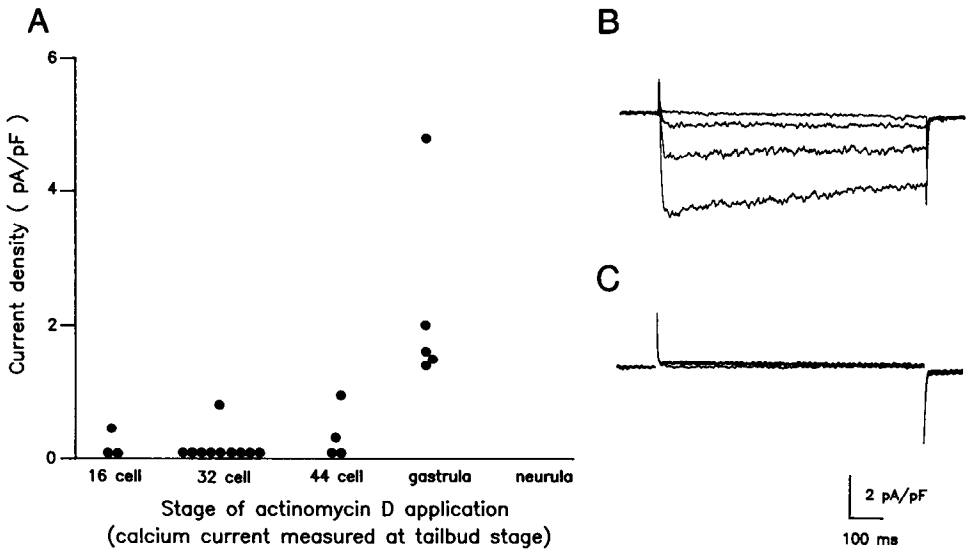

Figure 2. Effect of actinomycin D on Ca^{2+} current development. *A*, Peak Ba^{2+} current density recorded from single muscle-lineage cells at the tailbud stage plotted versus the stage at which actinomycin D exposure began. The average Ca^{2+} current density at tailbud stage in normally developing embryos, without drug treatment, was −13.4 pA/pF ($n = 8$). *B*, Recording from tail-bud-stage muscle cell treated with actinomycin D at gastrula stage. *C*, Recording from tailbud-stage muscle cell treated with actinomycin D at 32-cell stage. Currents were evoked from $V_h = -60$ mV by voltage steps to −40, −20, −10, and +10 mV in *B* and *C*. External solution was Ba-ASW, and the pipette solution was Cs-int.

their Fig. 3). Figure 5, *B* and *C*, shows I_{IR} records recorded at gastrula and tailbud stages, respectively, in embryos treated with actinomycin D at the 32-cell stage. When protein synthesis was blocked with emetine between 32-cell and gastrula stages, inwardly rectifying K^+ current disappeared normally ($n = 18$; data not shown). The apparently normal developmental disappearance of the inward rectifier could be an artifact of inhibition of protein synthesis and a high channel turnover rate. To control for this, we exposed embryos to emetine for equivalent periods of time at earlier stages when no loss of inward rectifier is seen normally. Under these conditions, the inward rectifier density remained approximately normal, indicating that turnover rate of this channel is at least longer than 6–8 hr.

Comparison of Ca^{2+} channel properties in muscle cells and oocytes

We compared the properties of Ca^{2+} channels that appear in muscle-lineage cells after gastrulation with those present at much lower density in the egg. In other tunicates (*Halocynthia roretzi* and *H. aurantium*), the Ca^{2+} current in the differentiated, cleavage-arrested embryo differs from that in the egg in both permeability sequence and inactivation mechanism (Hirano and Takahashi, 1984). The *Boltenia* Ca^{2+} channel in the egg (Hice, 1988) is similar to the mature *Halocynthia* Ca^{2+} channel: Both have the permeability sequence $Ba^{2+} > Sr^{2+} > Ca^{2+}$ and both have inactivation dependent on Ca^{2+} entry rather than on voltage. Figures 6 and 7 compare voltage dependence, permeability sequence, and inactivation mechanism for the *Boltenia* oocyte and muscle Ca^{2+} channels.

Figure 6*A* shows current–voltage relations for Ca^{2+} currents from an oocyte and a tailbud-stage muscle-lineage cell. Currents were measured in Ca-ASW external solution and Cs-int pipette solution. For comparison, we selected an oocyte and a muscle cell with similar peak current amplitudes. This emphasizes the dramatic difference in Ca^{2+} current density between these stages: The oocyte had a capacitance of 480 pF and a peak current density of −0.23 pA/pF in Ca-ASW (−0.43 pA/pF in Ba-ASW; this value is close to the mean value for oocyte current; Block and Moody, 1987); the muscle cell had a capacitance of 26 pF and a peak current density of −4.8 pA/pF in Ca-ASW (−17.9

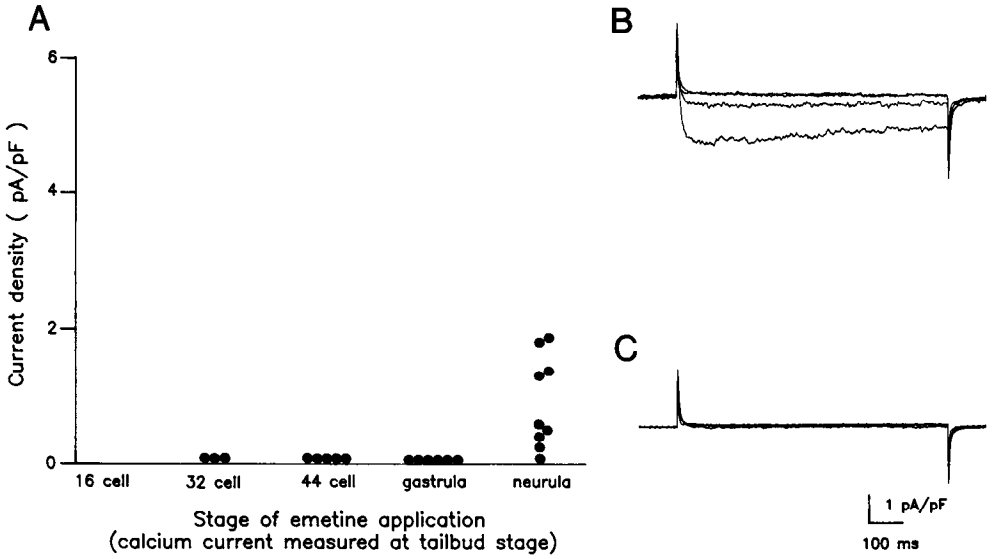

Figure 3. Effect of protein synthesis inhibition on Ca^{2+} current development. *A*, Peak Ba^{2+} current density recorded from single muscle-lineage cells at the tailbud stage plotted versus the stage at which emetine exposure began. *B*, Ca^{2+} records from a muscle cell treated with emetine at neurula stage. *C*, Ca^{2+} records from a muscle cell treated with emetine at the 32-cell stage. Currents were evoked from $V_h = -60$ mV by voltage steps to test potentials of −40, −20, −10, and +10 mV in *B* and *C*. External solution was Ba-ASW, and the pipette solution was Cs-int.

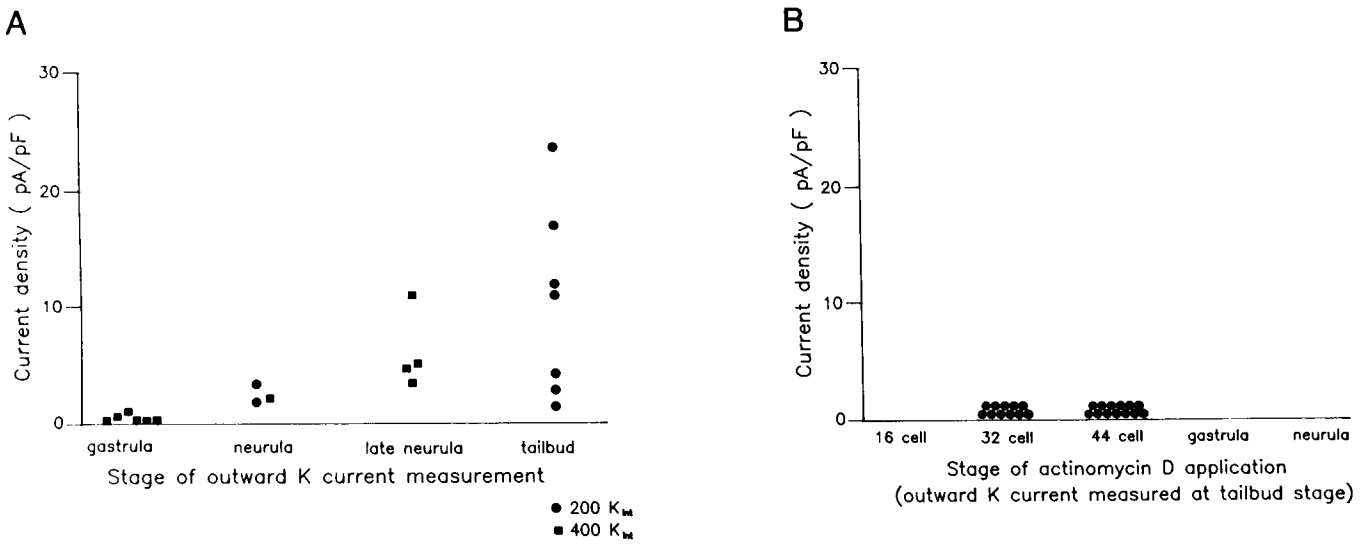

Figure 4. Delayed outward K^+ current development is sensitive to RNA synthesis blockers. *A,* Development of delayed outward K^+ current. Currents were measured at +40 mV from $V_h = -60$ mV. External solution was Sr-ASW, and the pipette solution was either 400 mM K-int (*squares*) or 200 mM K-int (*circles*). *B,* Current density measured at tailbud stage from embryos treated with actinomycin D at stages indicated on the *horizontal axis.*

pA/pF in Ba-ASW). The I–V relations for the two currents were very similar: Both currents were activated at potentials positive to −30 mV, with peak currents at +10 to +20 mV.

The permeability sequences for the two channels were also similar, though the muscle channel showed a significant higher Ba:Ca ratio than the egg channel. Figure 6, *B* and *C,* shows peak currents through Ca^{2+} channel from an oocyte (*B*) and from a muscle-lineage cell at the early tail-bud stage (*C*) as the permeant divalent is changed between Ca^{2+}, Sr^{2+}, and Ba^{2+} at a concentration of 50 mM. The permeability sequences calculated from such experiments (for details, see Fig. 6 caption) were oocyte: Ba^{2+} (2.29 ± 0.36) > Sr^{2+} (1.60 ± 0.2) > Ca^{2+} (1; $n = 3$); and muscle: Ba^{2+} (3.4 ± 0.21) > Sr^{2+} (1.63 ± 0.32) > Ca^{2+} (1; $n = 4$). Substitution of Ba^{2+} for Ca^{2+} in the external solution produced a shift in the peak of the *I–V* relation for the muscle-lineage current (−15 ± 7.1 mV; $n = 4$) but not for the oocyte current (−2.5 ± 2.9 mV; $n = 3$). Substitution of Sr^{2+} for Ca^{2+} produced no shifts in either egg or muscle. This difference in shift for Ba^{2+} between oocyte and tailbud stages could account for the apparent difference in Ba:Ca permeability ratio by changing the relationship between bulk solution Ba^{2+} concentration and the Ba^{2+} concentration at the channel mouth (see Ohmori and Yoshii, 1977).

Inactivation properties of Ca^{2+} channel in many cell types depend on the ionic species acting as current carrier and the magnitude of the current passing through the channel. Figure 6*B* shows that at both stages, oocyte and tailbud, inactivation of the current was greatly slowed when either Ba^{2+} or Sr^{2+} replaced Ca^{2+} as the charge carrier. This implies that inactivation is dependent on Ca^{2+} entry at both stages. Figure 7 shows a more rigorous test (see Brehm and Eckert, 1978). Inactivation versus voltage relations are shown for Ca^{2+} currents at oocyte and tailbud stages measured using the experimental protocol shown in Figure 7*B*. The Ca^{2+} current was measured at +20 mV after a 400-msec prepulse to different voltages. When the Ca^{2+} current during the prepulse was small (trace 1), the Ca^{2+} current mea-

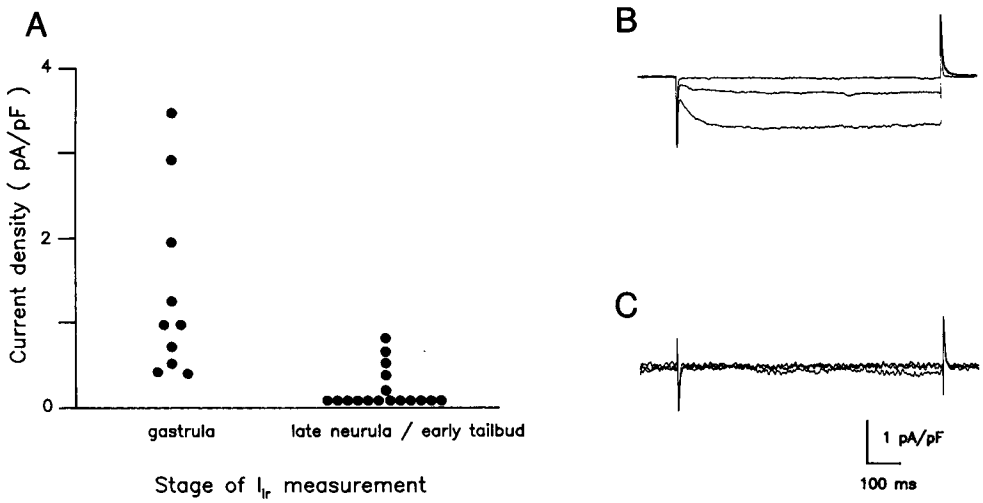

Figure 5. Effect of actinomycin D on the disappearance of the inwardly rectifying K^+ current (I_{IR}). *A,* Inward rectifier current density from single muscle-lineage cells at gastrula and late neurula/early tailbud stages from embryos treated with actinomycin D starting at the 32-cell stage. Current density was measured at −130 mV from $V_h = -50$ mV. External solution was 50 Sr-ASW, and the pipette solution was 200 mM K-int. The current disappeared normally in these embryos (see Fig. 1). *B* and *C,* Recordings from muscle cells at gastrula (*B*) and tailbud (*C*) stages from embryos exposed to actinomycin D starting at the 32-cell stage. In both *B* and *C,* currents were measured from $V_h = -50$ mV at test potentials of −80, −90, and −100 mV.

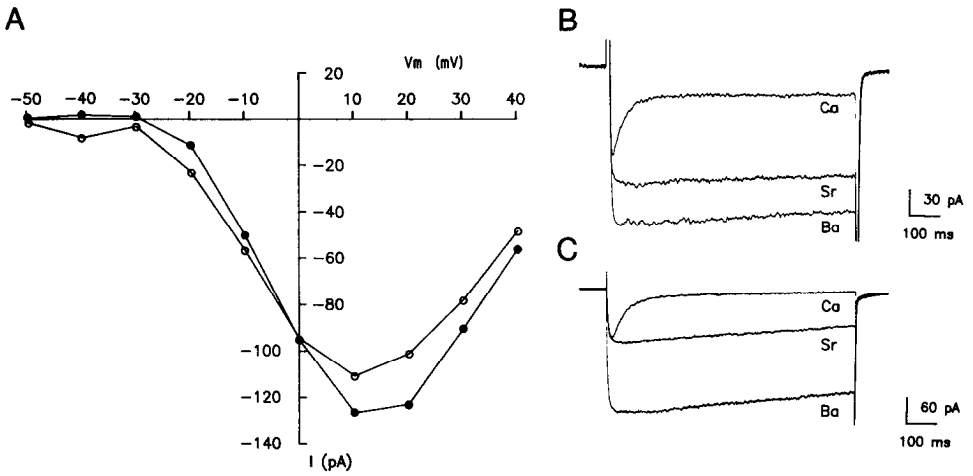

Figure 6. Comparison of properties of Ca^{2+} currents in oocyte and muscle cell. *A*, Current–voltage relation for an oocyte (*open circles*) and a muscle cell (*solid circles*). Internal solution was Cs-int, and external solution was Ca-ASW. *B* and *C*, Effect of isomolar substitution of Ca^{2+}, Sr^{2+}, and Ba^{2+} on current amplitude and inactivation for an oocyte (*B*) and a muscle cell at tail-bud stage (*C*). Currents were measured at V_t = +20 mV from V_h = −60 mV. Internal solution was Cs-int, and external solutions were either Ca-ASW, Sr-ASW, or Ba-ASW. (Note that the permeability sequences in the text were calculated from peak currents, whereas these currents are shown at the same voltage for the three ions.)

sured during the subsequent test pulse was large. The Ca^{2+} current measured with the test pulse reached a minimum when the Ca^{2+} influx during the prepulse reached a maximum (around +20 mV; trace 2). At higher voltages where the prepulse evoked smaller Ca^{2+} current, the Ca^{2+} current at the test pulse increased again (trace 3). Thus, test-pulse Ca^{2+} current is inversely related to Ca^{2+} entering the cell during the prepulse.

Discussion

The differential pigmentation of muscle-lineage cells in the ascidian *Boltenia villosa* allows them to be identified readily at the earliest stages of differentiation. This has allowed us to follow the development of their electrical properties throughout early embryogenesis. In previous work (Simoncini et al., 1988), we have shown that the muscle-lineage cells differentiate electrophysiologically from cells of other lineages just after gastrulation by expressing large Ca^{2+} and delayed K^+ currents during a period of just a few hours. At the same time, they eliminate the inwardly rectifying K^+ current present since fertilization, an event that occurs in cells of all lineages. In the present paper, we have shown that the appearance of Ca^{2+} and delayed K^+ currents in these cells after gastrulation depends on the products of RNA and protein synthesis, whereas the disappearance of the inward rectifier does not. The sensitive period of Ca^{2+} current development for inhibition of protein synthesis followed that of RNA synthesis by several hours. The appearance after gastrulation of these muscle-specific ion channels is similar to that of another muscle-specific protein, AChE. AChE expression is sensitive to inhibition of RNA synthesis just prior to gastrulation, which corresponds to the time when functional RNA for the esterase appears in the embryo (Meedel and Whittaker, 1983). Our results differ from those for endoderm-specific alkaline phosphatase, however. For this protein, which also first appears after gastrulation, the lack of an actinomycin-sensitive period (Whittaker, 1977) indicates that this protein is derived from maternal RNA, which is segregated into endoderm-lineage cells early in embryogenesis and is subject to later translational control.

Our results are unlikely to be due to nonspecific effects of the drugs on either the channels themselves or generally on development. Drugs were removed from the solution just before currents were recorded. For the Ca^{2+} current, a clear sensitive period was observed, and drug treatment after that period but still several hours before actual appearance of the current permitted the current to appear. Finally, the disappearance of the K^+ inward rectifier occurred normally when either RNA or protein synthesis was blocked. Our results do not, however, prove that the newly synthesized RNA codes for the channels themselves. The data are equally consistent, for example, with the synthesis of RNA that codes for a protein that activates translation of a preformed channel mRNA. This question can best be resolved by a direct measurement of Ca^{2+} and K^+ channel mRNA, either by hybridization or by oocyte expression assays, as has been done on a muscle cell line that can be induced to differentiate in culture by mitogen withdrawal (Caffrey et al., 1987, 1989).

The Ca^{2+} currents that appear in muscle-lineage cells after gastrulation are not the first Ca^{2+} currents to be present in the embryo. Very low-density Ca^{2+} currents can be recorded from the oocyte before fertilization and in all blastomeres through the 8-cell stage (Block and Moody, 1987); these currents disappear by the time of gastrulation and reappear in muscle-lineage cells a few hours later at much higher density than that at earlier stages. Because the Ca^{2+} currents present at early and late stages show such different cell specificity and density, we compared their permeability, kinetics, and voltage dependencies. The currents were similar in voltage dependence of activation, in the Ca^{2+} dependence of their inactivation, and qualitatively in their permeability sequence among divalents. These results differ from those obtained in another ascidian (Hirano and Takahashi, 1984), in which the Ca^{2+} current in the differentiated, cleavage-arrested embryo differed in both permeability sequence and inactivation mechanism from the oocyte current. This is likely to be a true species difference rather than an effect of cytochalasin treatment, because it is the oocyte currents, not the embryonic currents, that differ between the species. In our experiments, however, the oocyte and muscle Ca^{2+} currents did differ quantitatively in permeability ratio: The Ba^{2+}:Ca^{2+} ratio is higher in muscle (3.4 ± 0.21) than in the oocyte (2.29 ± 0.36). Correlated with this difference is the fact that equimolar substitution of Ba^{2+} for Ca^{2+} produced a 15-mV negative shift in the *I–V* relation in muscle but not in the oocyte. This suggests that the apparent difference in conductance ratios is caused by a difference in surface charge binding by Ba^{2+} between the two stages. Both negative shift and the increased Ba:Ca conductivity ratio in the muscle can be explained if Ba^{2+} is less effective at neutralizing fixed negative surface charges than is Ca^{2+}. Thus, at equal bulk solution concentrations of the two ions, the Ba^{2+}

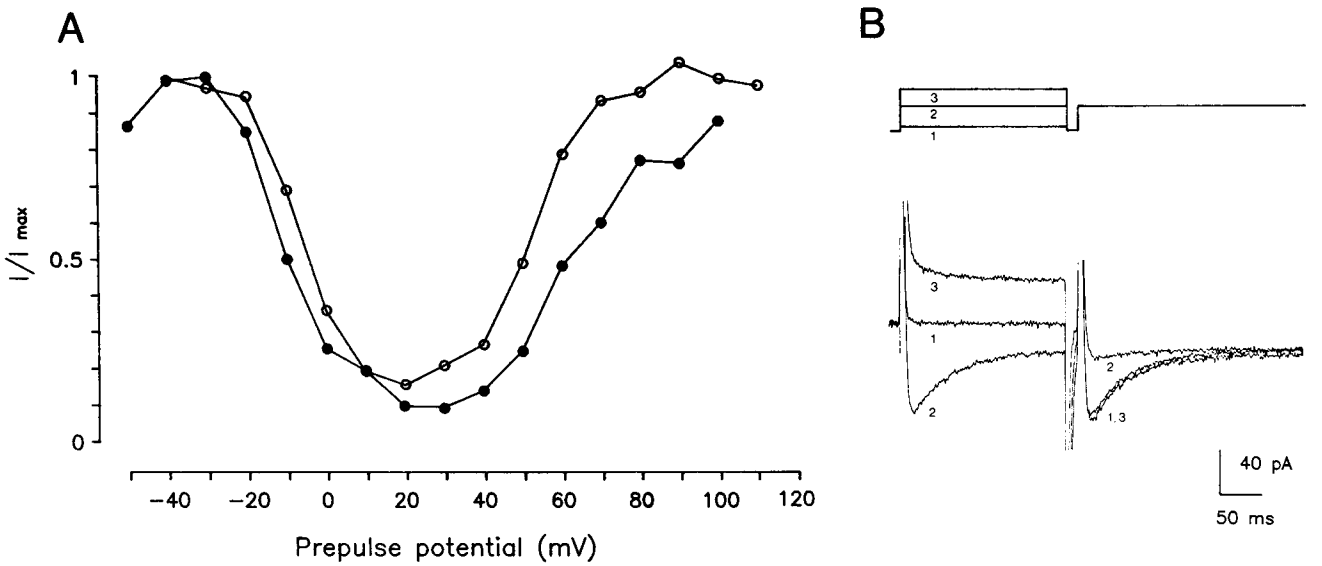

Figure 7. Calcium dependence of inactivation for Ca^{2+} channels in oocyte and muscle cell. *A*, Inactivation versus voltage relation for an oocyte (*open circles*) and a muscle cell at tailbud stage (*solid circles*). Currents were measured at a V_t = +20 mV from prepulse potentials between −40 and +110 mV and normalized. Internal solution was Cs-int, and external solution was Ca-ASW. *B*, Currents recorded in an oocyte with the experimental paradigm used to generate the plot in *A*. Prepulses are shown to −40 (*1*), + 20 (*2*), and +120 mV (*3*), with the test pulse to +20 mV.

concentration at the channel itself may be higher than the Ca^{2+} concentration. This effect does not occur in the oocyte. Thus, the ion pore of the oocyte and muscle channels may well have equal conductance ratios for the two ions, but the muscle channel appears to conduct Ba^{2+} more efficiently because it may be exposed to higher Ba^{2+} concentrations. This conclusion relies on the assumption that surface charge measured by a gating property (voltage dependence) pertains to charge experienced at the channel mouth. It is notable that the appearance of Ca^{2+} channels in muscle requires new RNA synthesis even though an apparently identical channel is expressed at low levels in the oocyte.

The progression of electrical properties in *Boltenia* muscle-lineage cells shows some interesting differences from that in other muscle. First is the lack of appearance of an Na^+ current, a type of which is expressed during vertebrate muscle development (Caffrey et al., 1989). This is not surprising, because most invertebrate muscle cells lack a voltage-dependent Na^+ current (but see Schwartz and Stuehmer, 1984) and rely on Ca^{2+} entry through Ca^{2+} channels in the surface membrane for contraction. Our results are consistent with reports that mature larval tunicate muscle has a purely Ca-dependent action potential (Ohmori and Sasaki, 1977) and with the absence of an Na^+ current in muscle-lineage cells in differentiated, cleavage-arrested ascidian embryos of other species (Okado and Takahashi, 1990). Second, the delayed K^+ current appears at approximately the same stage as the Ca^{2+} current in our experiments, whereas in some myogenic cell lines it is constitutively expressed independent of the state of differentiation (Caffrey et al., 1987). This, however, may reflect a property peculiar to the cell lines, because the action potential in developing chick muscle shows a greatly shortened duration later in development, implying the appearance of a delayed K^+ current (Kano, 1975). Finally, the disappearance of the inwardly rectifying K^+ current has not been reported in muscle development, though a decrease in the resting K^+:Cl^- permeability ratio has (Heathcote, 1989). In other ascidian embryos, differentiating without cell division in the presence of cytochalasin B, muscle-lineage cells have an inward rectifier at a later stage (Hirano et al., 1984). It is possible that the reduction we see in the inward rectifier is a transient one, because an inward rectifier is present in most mature muscle cells.

The coordinate appearance of Ca^{2+} currents and elimination of the inward rectifier in *Boltenia* muscle just after gastrulation raises an interesting possibility. The loss of the inward rectifier may create a cell that has an unstable resting potential and is thus prone to generate spontaneous action potentials resulting in substantial Ca^{2+} entry. Because these changes occur relatively early in development, before the appearance of contractility and innervation, it is possible that spontaneous activity and Ca^{2+} influx influence later events in muscle differentiation. It has been reported in several instances that various aspects of muscle differentiation depend on spontaneous activity, Ca^{2+} entry, or the presence of external Ca^{2+} (see, e.g., Cerny and Bandman, 1986; Heathcote, 1989; see also Holliday and Spitzer, 1990). We are currently investigating these questions.

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