Na^+ channel mis-expression accelerates K^+ channel development in embryonic *Xenopus laevis* skeletal muscle

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1. The normal developmental pattern of voltage-gated ion channel expression in embryonic skeletal muscle cells of the frog *Xenopus laevis* was disrupted by introduction of cloned rat brain Na^+ channels.

2. Following injection of channel mRNA into fertilized eggs, large Na^+ currents were observed in muscle cells at the earliest developmental stage at which they could be uniquely identified. Muscle cells normally have no voltage-gated currents at this stage.

3. Muscle cells expressing exogenous Na^+ channels showed increased expression of at least two classes of endogenous K^+ currents.

4. This increase in K^+ current expression was inhibited by the Na^+ channel blocker tetrodotoxin, suggesting that increased electrical activity caused by Na^+ channel mis-expression triggers a compensatory increase in K^+ channel expression.

5. Block of endogenous Na^+ channels in later control myocytes retards K^+ current development, indicating that a similar compensatory mechanism to that triggered by Na^+ channel mis-expression operates to balance Na^+ and K^+ current densities during normal muscle development.

The process by which electrically excitable cells attain the appropriate mature pattern of voltage-gated ion channels is complex. Highly stereotyped temporal patterns of voltage-gated ion channel expression have been identified in developing muscle cells (Spruce & Moody, 1992; Broadie & Bate, 1993) and neurons (O'Dowd, Ribera & Spitzer, 1988), suggesting that the precise order in which functional channels appear in a given cell is both strictly regulated and critical to normal development. However, it has not previously been possible to test the importance of such a developmental sequence directly by altering the pattern of channel expression in a developing cell.

Embryonic skeletal muscle cells of the frog *Xenopus laevis* express both inward rectifier (I_{IR}) and sustained outward K^+ currents (I_K) before Na^+ currents (Spruce & Moody, 1992), suggesting that electrical activity is tightly limited during early myogenesis. To test the functional significance of this sequence of K^+ and Na^+ channel expression, we reversed it, forcing Na^+ currents to appear first. This was achieved by injecting cloned Na^+ channel mRNA into the fertilized egg.

A preliminary report of this work has previously been given (Linsdell & Moody, 1994).

METHODS

Fertilization and culture of embryos

Eggs were extruded from freely moving *Xenopus laevis* primed with injections of pregnant mare serum gonadotropin (50 IU; Calbiochem Corp., La Jolla, CA, USA) and human chorionic gonadotropin (1000 IU; Serono Laboratories Inc., Randolph, MA, USA), and fertilized with a portion of macerated testis. Testes were removed surgically from frogs anaesthetized using 0·2% Tricaine (MS-222, Sigma) in iced water. Following the removal of the first testis, frogs were allowed to recover in isolation. Frogs recovering from surgery were monitored closely for any signs of discomfort; none were noted. Two to three weeks later the second testis was removed, after which the frogs were killed by cervical section. Fertilized eggs were dejellied with 2% cysteine solution (Sigma) and reared at 16–18 °C in 10% modified Barth's solution (mM: 8·8 NaCl, 0·1 KCl, 0·24 $NaHCO_3$, 0·082 $MgSO_4$, 0·033 $Ca(NO_3)_2$, 0·1 $CaCl_2$, 1 Hepes, pH 7·4). Embryos were staged according to Nieuwkoop & Faber (1967) and dissected at the neural plate stage (Stage 15), approximately 18 h after fertilization, which is the earliest stage at which we could identify muscle cells on the basis of their morphology and location within the embryo. *In vivo*, innervation of *Xenopus* mesoderm begins around 5–6 h after Stage 15 (approximately Stage 21–22) and spontaneous movements are first seen around 12 h after Stage 15 (approximately Stage 26–27).

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To obtain myocytes, the dorsal portion of the Stage 15 embryo was excised into Ca^{2+}–Mg^{2+}-free solution (mM): 52·8 NaCl, 0·7 KCl, 0·4 EDTA, 5 Tris, pH 7·5) containing 1 mg ml^{-1} papain (Calbiochem) to facilitate removal of the endoderm. The mesoderm was then stripped away from the neuroectoderm and placed in fresh Ca^{2+}–Mg^{2+}-free solution to obtain dissociated cells, which were triturated onto tissue culture plastic dishes (Falcon 3001; Becton Dickinson Co., Oxnard, CA, USA) containing Danilchik's medium (mM): 53 NaCl, 27 sodium isothionate, 15 $NaHCO_3$, 4·5 potassium gluconate, 1 $MgSO_4$, 1 $CaCl_2$, pH 8·3). These conditions have previously been shown to allow normal development of these muscle cells in culture (Spruce & Moody, 1992).

RNA injection

In some experiments, the complement of ion channels present in early muscle cells was altered by expression of cloned channel mRNA. In these experiments, fertilized eggs were injected in the vegetal hemisphere with 40 nl RNA solution, which contained either Na^+ channel α-subunit (5 ng μl^{-1}) plus β_1-subunit (25 ng μl^{-1}) mRNAs, or K^+ channel mRNA (5 ng μl^{-1}). As a control, the same concentration of Na^+ channel β_1-subunit mRNA alone, which does not encode a functional channel, was injected into some embryos. The mRNAs used were those encoding the rat brain type IIA Na^+ channel α-subunit (Auld *et al.* 1988), rat brain Na^+ channel β_1-subunit (Isom *et al.* 1992), and mouse brain Kv1·1 delayed rectifier-type K^+ channel (Tempel, Jan & Jan, 1988). Embryos injected with channel mRNA developed normally and were dissected at Stage 15 as described above.

Electrophysiology

Currents were recorded with the whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) using a List Electronic (Darmstadt, Germany) EPC-7 amplifier, filtered at 1 kHz using an 8-pole Bessel filter, and acquired at 2–33 kHz using pCLAMP software. During recordings, the extracellular solution was Danilchik's medium (see above) and the internal (pipette) solution contained (mM): 80 potassium aspartate, 10 KCl, 10 NaCl, 2 $MgCl_2$, 2 EGTA, 3 glucose, 2 theophylline, 2 MgATP, 0·1 cAMP, 10 Hepes, pH 7·4. Measurement of current amplitudes following subtraction of linear leak currents was as described previously (Spruce & Moody, 1992). Measured current amplitudes were divided by cell capacitance (measured as described previously; Moody & Bosma, 1985) to calculate current densities. Recordings were made at room temperature, 20–23 °C. Values are expressed as means ± S.E.M.

RESULTS

Expression of exogenous Na^+ currents in embryonic myocytes

Following coinjection of mRNAs encoding rat brain Na^+ channel α- and β_1-subunits into fertilized eggs, large Na^+ currents were recorded from myocytes dissociated at Stage 15 (Fig. 1*A*). Such Na^+ currents were observed in 98% of these myocytes in the first 2 h after Stage 15 (mean current density, 79·4 ± 20·4 pA pF^{-1}, $n = 40$). In contrast, Na^+ currents were never observed at this time in myocytes from uninjected control embryos ($n = 58$; Fig. 1*A*), embryos injected with β_1-subunit mRNA alone ($n = 18$), or embryos injected with Kv1·1 K^+ channel mRNA ($n = 13$). The Na^+ current seen in these cells following Na^+ channel mRNA injection showed similar kinetics and voltage dependence of activation and inactivation to those previously reported for cloned type II A channels expressed in other cell types (Isom *et al.* 1992; West, Scheuer, Maechler & Catterall, 1992). This current was clearly distinguishable from the endogenous Na^+ current, which is first seen in control myocytes 8 h after Stage 15. Exogenous Na^+ channels were half-maximally activated at a membrane potential 8·4 mV more positive and half-inactivated 12·9 mV more negative than endogenous Na^+ channels. The exogenous current also inactivated more rapidly, with a mean time constant of inactivation of 0·27 ± 0·02 ms ($n = 23$) at 0 mV, compared with 0·47 ± 0·04 ms ($n = 22$) for the endogenous Na^+ current. Thus, foreign ion channels can be introduced into the membrane of developing muscle cells by mRNA injection into the fertilized egg.

Early Na^+ channel expression increases K^+ channel expression

Myocytes expressing exogenous Na^+ currents also showed increased densities of the endogenous K^+ currents I_K and I_{IR} (Fig. 1*B* and *C*; Fig. 2). I_K and I_{IR} were identified according to previously described criteria (Spruce & Moody, 1992). All of the currents shown in Fig. 1*B–E* were abolished on replacement of internal K^+ ions with Cs^+, identifying them as K^+ currents. As previously shown for control cells (Spruce & Moody, 1992), I_K in Na^+ channel expressing cells was initially very rapidly activating (Fig. 1*B*), with a more slowly activating component appearing at later times (Fig. 1*D*).

I_K in Na^+ channel-expressing cells was already substantially upregulated by Stage 15, the earliest time examined, whereas the largest effects on I_{IR} were seen starting at 2 h after Stage 15 (Fig. 2). Since muscle cells cannot be uniquely identified before Stage 15, we could not determine how early in development Na^+ channel mis-expression affected I_K. At 6 h after Stage 15, when most control myocytes showed both I_K and I_{IR}, the amplitudes of both currents were still larger in Na^+ channel-expressing myocytes (Fig. 1*D* and *E*; Fig. 2). By this stage in control myocytes, I_K had become more slowly activating in many cells (Fig. 1*D*). The appearance of this slowly activating component of I_K also appeared to be accelerated by Na^+ channel expression, as it could be identified in 72% (13/18) of Na^+ channel-expressing cells at 6 h after Stage 15, compared with 50% (10/20) of control cells. Acutely blocking the Na^+ current by applying 1 μM tetrodotoxin (TTX; Sigma) had no effect on I_K or I_{IR}, indicating that they are not directly influenced by Na^+ influx (e.g. Kameyama, Kakei, Sato, Shibasaki, Matsuda & Irisawa, 1984). Thus early Na^+ channel expression appears to increase the density of functional K^+ channels

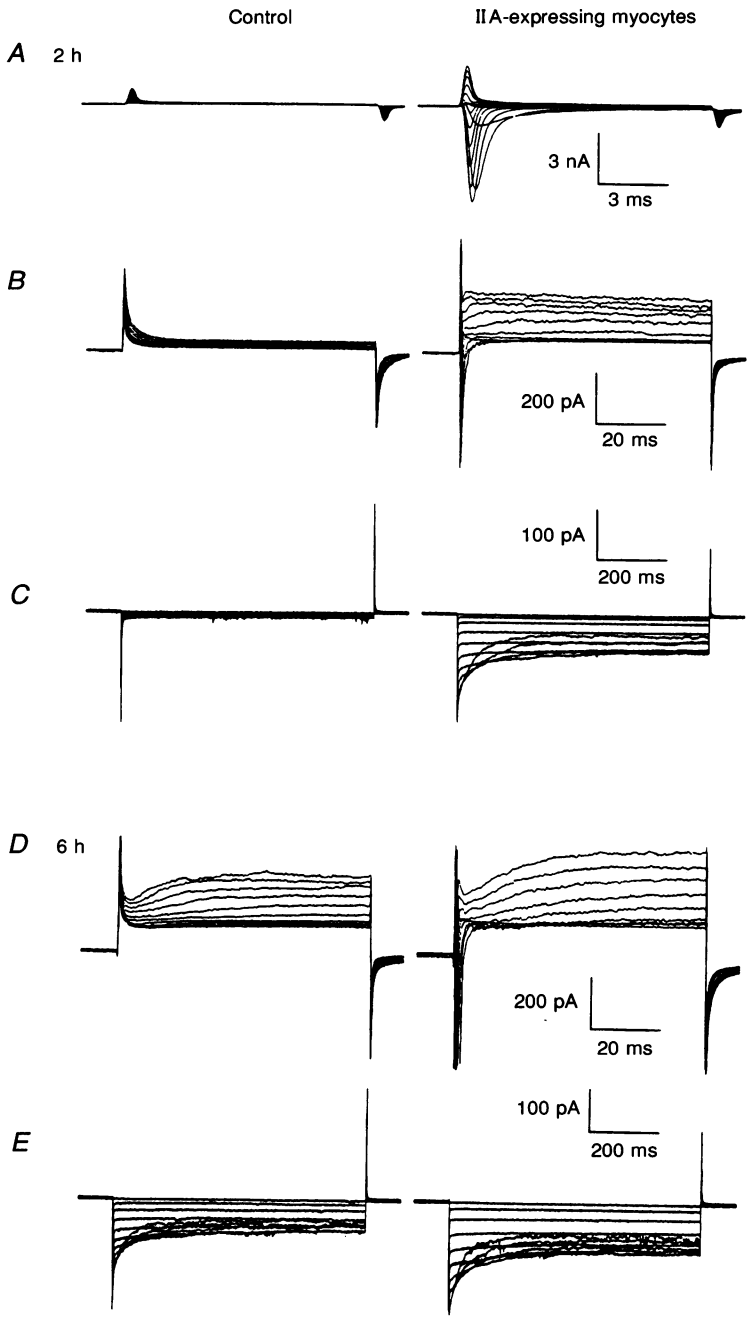

Figure 1. Whole-cell currents recorded from *Xenopus* myocytes dissociated from control embryos (left) and from embryos grown from eggs injected with Na^+ channel mRNA (right)
Recordings were made 2 (*A–C*) or 6 h (*D* and *E*) after dissociation at Stage 15. *A*, large, transient Na^+ currents are seen in myocytes following Na^+ channel mRNA injection into the egg at a time at which the endogenous Na^+ current is never seen. Holding potential, −100 mV; voltage steps to between −70 and +80 mV in 10 mV increments. These Na^+ channel-expressing cells also express sustained outward (I_K; *B*) and inward rectifier (I_{IR}; *C*) K^+ currents at much higher densities than control myocytes at the same stage. Note the smaller current scale than in *A*. At later times, when I_K (*D*) and I_{IR} (*E*) are both seen in control myocytes, the amplitudes of both are still greater in Na^+ channel-expressing cells. In *B* and *D*, the holding potential is −100 mV and steps are to between −50 and +40 mV; in *C* and *E*, the holding potential is −60 mV and steps are to between −60 and −150 mV.

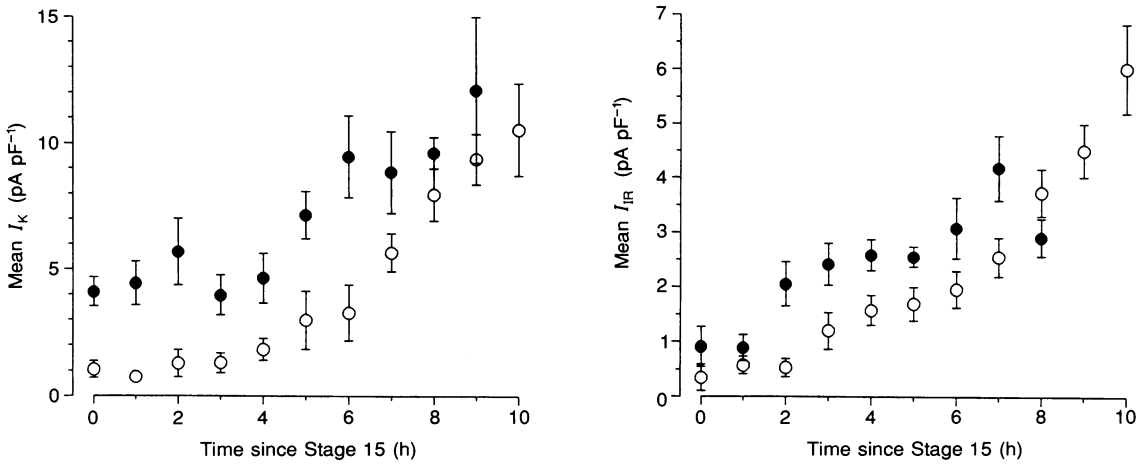

Figure 2. Development of K^+ currents in control and Na^+ channel-expressing myocytes
○, control; ●, Na^+ channel-expressing myocytes. Each point shows the mean (± S.E.M.) of data from 4–23 myocytes, grouped into 1 h bins relative to Stage 15. The increase in I_K due to Na^+ channel expression is apparent at Stage 15, whereas the increase in I_{IR} appears to be more delayed. The current densities in Na^+ channel-expressing cells were significantly greater than in control cells at all time points between 0 and 8 h after Stage 15 for I_K, and from 2 to 7 h after Stage 15 for I_{IR} ($P < 0.05$, Student's one-tailed t test). Mean cell capacitance was not significantly different in Na^+ channel-expressing and control cells at all stages.

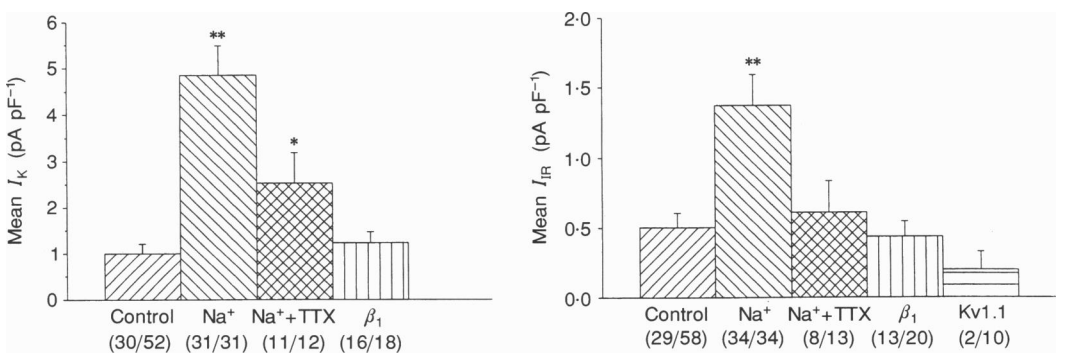

Figure 3. Mean K^+ current densities between 0 and 2 h after Stage 15
Current densities are shown for control, Na^+ channel-expressing (Na^+), Na^+ channel-expressing raised in TTX (Na^+ + TTX), Na^+ channel β_1-subunit alone-expressing (β_1), and Kv1·1 channel-expressing (Kv1·1) myocytes. Error bars represent 1 S.E.M. The values in parentheses beneath each column represent the number of cells expressing any identifiable I_K or I_{IR} as a fraction of the total number of cells sampled; both of these K^+ currents were present in all Na^+ channel-expressing myocytes. The endogenous outward K^+ current in Kv1·1-expressing cells was masked by the large current through Kv1·1 channels, which had a mean density of 26·5 ± 4·2 pA pF^{-1} ($n = 15$). Significant increases in current density over controls are shown: ** $P < 0.001$; * $P < 0.05$ (Student's one-tailed t test). I_K density in Na^+ channel-expressing cells was significantly reduced by culturing embryos in TTX ($P < 0.0025$). I_{IR} density was significantly less in Kv1·1-expressing cells than controls ($P < 0.025$), suggesting that exogenous K^+ channel expression may reduce excitability during myogenesis, thereby reducing endogenous K^+ channel expression. TTX-treated embryos were grown from fertilization until Stage 15 in the presence of 1 μM TTX.

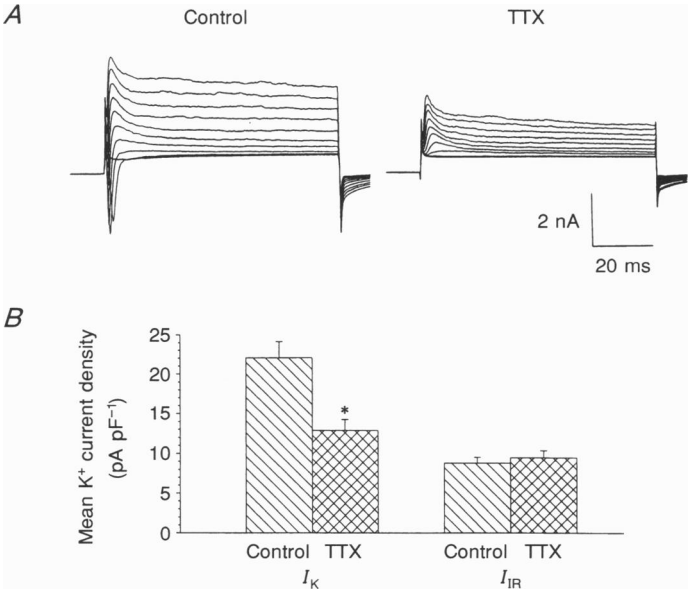

Figure 4. Chronic TTX exposure prevents normal K^+ current maturation
A, depolarization-activated currents in normal myocytes cultured from Stage 15 for 24 h in the absence (left) and presence (right) of 1 μM TTX. TTX eliminates the Na^+ current and reduces the amplitude of I_K. This effect appears to be due to a reduction in functional K^+ channel expression, since I_K amplitude was not increased on applying TTX-free solution. Both control and TTX-cultured myocytes had developed a transient component to the outward current by this stage. Voltage protocol as in Fig. 1*B*. *B*, mean I_K and I_{IR} densities in control and TTX-cultured (TTX) myocytes, measured 18–24 h after dissociation at Stage 15. Mean I_K density was significantly reduced by culturing in TTX (* $P < 0{\cdot}001$, Student's one-tailed t test), whereas mean I_{IR} density was not significantly different from control. Error bars represent 1 S.E.M.; $n = 23$–47.

in the cell membrane. Figures 2 and 3 summarize sustained outward and inwardly rectifying K^+ current densities at different stages in Na^+ channel expressing and control myocytes.

Functional Na^+ channels are necessary to increase K^+ channel expression

Culturing embryos in the presence of TTX eliminated the effect of Na^+ channel expression on I_{IR} and significantly reduced the effect on I_K (Fig. 3), indicating that expressed Na^+ channels must be functional in order to affect K^+ current density. Since TTX only partially reversed the effects of Na^+ channel expression on I_K density, it did not greatly affect the proportion of cells in which I_K could be identified (Fig. 3). These partial effects of TTX are most likely to be due to incomplete penetration of TTX into the embryo, since the epithelial seal of the *Xenopus* embryo presents a substantial diffusional barrier to even small molecules (Regen & Steinhardt, 1986).

Expression of Na^+ channel β_1-subunit mRNA alone, which does not code for a functional channel, or of the mouse delayed rectifier K^+ channel Kv1·1, did not increase endogenous K^+ channel density, indicating that this effect is specific to functional Na^+ channels. However, expression of the β_1-subunit alone appeared to increase the proportion of cells in which I_K could be identified, suggesting the possibility that the presence of Na^+ channel β_1-subunits may have some small effect on K^+ current development. It is clear, however, that most of the effects of Na^+ channel expression on I_K and I_{IR} are mediated by functional Na^+ channels in the cell membrane, probably due to an increase in excitability during early embryogenesis. We believe these effects are most likely to be due to increased excitability in the muscle cells themselves, although we cannot rule out the possibility that contact between presumptive muscle cells and Na^+ channel-expressing non-muscle cells in the early embryo could affect muscle K^+ current expression.

The role of Na^+ channels in normal K^+ current development

In cultured control myocytes there is normally an approximately 3-fold increase in the densities of both I_K and I_{IR} between 8 h after Stage 15, when the endogenous Na^+ current first appears, and 24 h (P. Linsdell & W. J. Moody, unpublished observation). To test whether the endogenous Na^+ current plays a role in this normal developmental increase in K^+ current density, myocytes dissociated from Stage 15 control embryos were cultured for 18–24 h (to approximately Stage 30–33) in the presence

and absence of TTX. I_{IR} was unaffected by TTX, but I_K was significantly smaller in TTX-cultured myocytes (Fig. 4). Thus the presence of endogenous functional Na^+ channels is necessary for the later development of I_K.

DISCUSSION

Our results indicate that functional Na^+ channels upregulate K^+ current expression in developing muscle cells, both during normal development and following abnormally early Na^+ channel expression. The fact that Na^+ channels must be functional to have this effect suggests that the expression of muscle K^+ channels may be sensitive to electrical activity, as is the case, for example, for the mammalian skeletal muscle Na^+ channel (Sherman & Catterall, 1984). The increase in K^+ channel expression seen would tend to compensate for the excitatory effects of early Na^+ channel expression. In terms of directly opposing the excitatory effects of Na^+ channel activation, I_K is likely to be more effective than I_{IR}, and thus one might expect I_K expression to be more tightly coupled to Na^+ channel function than I_{IR}. Indeed, exogenous Na^+ channel expression had a stronger effect on I_K (Fig. 2) that was more difficult to reverse with TTX (Fig. 3), and blocking endogenous Na^+ channels reduced later I_K, but not I_{IR}, development (Fig. 4). The responses caused by precocious Na^+ channel expression may thus reflect the need to control strictly excitability throughout myogenesis, which is seen both in the normal sequence in which K^+ current expression precedes Na^+ current expression, and in the coupling between endogenous Na^+ channel activity and K^+ current density later in development.

The increase in K^+ current density seen in myocytes expressing exogenous Na^+ channels could result from precocious K^+ channel expression or increased K^+ channel expression over the normal developmental time course. Although our data cannot distinguish between these two possibilities, the data in Fig. 2 suggest the former. However, since we are unable to uniquely identify muscle cells before Stage 15, we are unable to test this directly.

Our results indicate that even very specific disruptions of one channel type during development can affect the level of expression of several different channels. Thus any effects attributed to the specific alteration of the activity of one channel type, particularly during development, may not be due directly to changes in the activity of the targeted channels.

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