Development and Subsequent Neural Tube Effects on the Excitability of Cultured *Xenopus* Myocytes[1]

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Abstract

We examined both (1) the development of electrical excitability in cultured *Xenopus* muscle over a period of 7 days, and (2) the effects of neural tube on the muscle action potential. During muscle development, delayed and anomalous rectification were present in most cases within 24 hr. The action potential was dependent on Na at all times examined, and the rate of rise of the action potential (V_{max}) increased substantially (seven-fold) from the first 2 days to 6 to 7 days *in vitro*, reflecting an increase in Na current density. In order to determine the mechanism for the increase in V_{max}, we examined single-channel Na currents using the gigaseal technique. Single-channel conductance (γ) did not increase substantially when measured using the patch clamp technique: $\gamma = 24$ pS at 1 to 2 days, and $\gamma = 28$ pS at 4 to 6 days. The channel open time at 14°C was 0.6 msec for 1- to 2-day-old cells and 0.5 msec in 4- to 6-day-old cells at a step potential 40 mV from rest. The time constant for current decay as well as the time-to-peak current also did not change over time. Thus, channel kinetics appear unchanged. The maximum inward current from summed records was statistically greater for older cells, and the frequency of patches displaying single-channel events increased from 75 to 98%. Thus, we conclude that during development *in vitro*, Na current density increases as a result of an increase in channel density without detectable alterations in single-channel properties.

Neural tube addition led to a further increase in V_{max} (twofold), even in muscle cells with no apparent nerve contact. Single channel analysis of cells in coculture revealed γ to be 28 pS in three cells displaying a single amplitude peak for individual Na currents. In the majority of cases (9/12), however, there appeared to be two classes of Na channels present which were difficult to separate. The larger conductance channel likely corresponds to the 28 pS class. The smaller channels, when present, did not contribute substantially to the population of events comprising the amplitude histogram. Other single-channel kinetic parameters also did not change. We, therefore, conclude that neural tube addition does not effect activation or inactivation kinetics but likely causes a further increase in channel density and possibly the induction of a second type of Na channel.

Embryonic nerve and muscle cells undergo a variety of changes in their membrane properties which allow them to function as mature, excitable cells. Both the distribution and functional characteristics of ionic channels can be modified during development. Some of these changes continue to occur in isolated cells developing in culture, but others depend upon extrinsic factors such as innervation. Voltage and chemically gated ion channels have been studied in a number of systems to determine how the properties of these channels are modified during development and subsequent innervation. One such channel, the acetylcholine receptor (AChR) on cultured *Xenopus* muscle, has been studied extensively in this laboratory (see Kidokoro et al., 1982). Both the open time and the conductance of these channels change during development and are altered upon addition of neural tube cells. The first part of this paper examines whether developmental changes occur in the channels involved in action potential (AP) generation in cultured *Xenopus* muscle. The second section investigates the effect of adding dissociated cells from the embryonic neural tube on the voltage-sensitive Na channel.

The AP of vertebrate excitable cells usually displays substantial changes as the cells mature. Young cells often respond passively when depolarized. In time, an AP develops and its maximum rate of rise (V_{max}) increases. In neurons (Baccaglini and Spitzer, 1977; Willard, 1980) and striated muscle (Kano et al., 1972; Kano, 1975; Kidokoro, 1975; Spector and Prives, 1977), there is a shift from the initial Ca-dependent spike to one that is Na-dependent (with an occasional residual Ca component). Delayed rectification usually is present from the time when a regenerative response is first observed. The above observations on changes in ionic dependence of the action potential suggest that increased inward current during spike generation can be accounted for by an addition of functional Na channels which have faster activation kinetics than Ca channels. In many cases, the Na channels appear to eventually replace Ca channels. In studies looking specifically at the development of Na channels in cultured chick (Frelin et al., 1981; Baumgold et al., 1983a, b; Strichartz et al., 1983) and rat (Sherman and Catterall, 1982, 1983) muscle, the density of these channels has been shown to increase over time. For chick muscle, the increase was accompanied by a concommitant increase in V_{max} and batrachotoxin-stimulated ^{22}Na flux, suggesting the addition of functional channels. Whether the functional characteristics changed in single Na channels has not been examined.

Tetrodotoxin (TTX) sensitivity of Na channels has been examined extensively during development and upon denervation. In rat muscle,

early in development (Harris and Marshall, 1973) and after denervation (Redfern and Thesleff, 1971; Harris and Thesleff, 1971), at least a portion of the Na channels become resistant to TTX. In the chicken and frog, however, denervation does not result in a TTX-resistant AP (Harris et al., 1973; Nasledov and Thesleff, 1974). Little has been done to examine whether denervation *in vivo* or the addition of nerve *in vitro* causes alterations in Na channel properties. Voltage-clamp analysis of denervated rat muscle (Pappone, 1980) showed that the only detectable change in TTX-sensitive macroscopic current was a shift to more negative potentials in the voltage dependence of activation and inactivation kinetics. In cultured chick muscle exposed to nerve extract, electrophysiological measurements suggested that Na channel density increased, although alterations in individual channel properties were not examined (Kano et al., 1979).

We have used intracellular and patch recording techniques to determine the developmental changes of voltage-gated channels in cultured *Xenopus* muscle and the subsequent effect of the addition of dissociated neural tube cells on the Na action potential in these muscle cells. Na channels carry inward current from the earliest time studied. The amount of current increases with time in culture, reflecting a change in Na channel density rather than alterations in either channel kinetics or unitary conductance. Neural tube addition induces a detectable increase in $\dot{V}_{max}$, but the analysis of individual Na channel currents is complicated. Most muscle cells exhibit at least two types of Na channels which were not easily separated. The addition of neural tube to muscle cultures seems to increase Na current by increasing the density of Na channels, possibly through the induction of a second channel type. The single channel properties of the major class of Na channels, however, was unaffected by neural tube addition.

Materials and Methods

Cultures

Myotomes from *Xenopus laevis* embryos were dissociated and cultured as described by Kidokoro et al. (1980) with some modifications. The dorsal portions of stage 18 to 27 embryos (Nieuwkoop and Faber, 1967) were isolated with dissecting needles in 60% (v/v) HEPES-buffered Dulbecco-modified Eagle's medium (with 5% horse serum (GIBCO), 50 μg/ml gentamycin (Shering) and 50 unit/ml mycostatin (GIBCO)). Myotomes were separated from the surrounding tissue in 0.1% collagenase (Worthington) in 67 mM NaCl, 1.5 mM KCl, 0.5 mM CaCl$_2$, and 8 mM HEPES-NaOH, pH 7.4. Individual muscle cells were obtained by incubating myotomes in 2 mM EDTA, 67 mM NaCl, 1.6 mM KCl, and 8 mM HEPES-NaOH (pH 7.4). Cells were then plated in 60% HEPES-buffered Dulbecco-modified Eagle's medium with either 0, 0.5, or 5% horse serum. After one day, the concentration of serum was reduced to 0.5% from 5%. For nerve-muscle cocultures, dissociated neural tube cells from stage 18 to 20 embryos were added to 1- to 3-day old muscle cultures. Experiments were done 2 to 4 days after adding the neural tube cells when the muscle cultures were 4 to 6 days old. Cultures were kept at room temperature (21–24°C).

Solutions

The recording solution for all experiments was 120 mM NaCl, 1.6 mM KCl, 10 mM CaCl$_2$, 8 mM HEPES-NaOH (pH 7.4). Tetrodotoxin, tetraethylammonium, and 4 aminopyridine were obtained from Sigma.

Electrophysiology

A single microelectrode (50 to 100 megohms) filled with 3 M potassium acetate and 10 mM potassium chloride was used to pass current and measure membrane potential via a bridge circuit in a Biodyne Electronics AM-1 amplifier. The voltage response was differentiated electronically. Voltage, dv/dt, and current records were then stored on a Racal Store 4DS tape recorder (10 kHz band width) for later analysis. Small hyperpolarizing pulses were used to determine the input resistance (R_{in}) and the membrane time constant (τ_m) before depolarizing the cell to elicit an AP. In 40% of the cases, the membrane potential was held at approximately −90 mV if they initially were below −80 mV. The current pulse experiments were done at room temperature (21–24°C).

Single-channel current recordings were made with Sylgard (Dow-Corning) coated electrodes (flint glass, 3 to 10 megohms) according to the method outlined by Hamill et al. (1981). Electrodes contained 120 mM NaCl, 1.6 mM KCl, 0.5 mM EGTA, and 8 mM HEPES-NaOH (pH 7.4) when recording from cell attached patches. Records were stored on tape directly from a Meyer and Renz MPCS-V3 patch clamp amplifier which contained a transient cancellation circuit. The fully compensated, unfiltered patch clamp current had a 10 to 90% rise time of 45 μsec.

Throughout the paper, voltage steps are reported as the displacement from the resting potential (for example, a 40-mv step depolarizes the patch membrane by 40 mV). In some cases, the membrane potential was estimated by rupturing the patch membrane and determining the voltage which produced zero current. Membrane potential was corrected for a liquid junction potential of 1.5 mV. In most patch clamp experiments, the cells were cooled to slow down the rates of channel opening and closing. Except where noted, the temperature was 14 ± 1°C (SD).

Data analysis

Cell size was measured to estimate specific membrane resistance (R_m) and capacitance (C_m). Photomicrographs were printed (× 1900) and the cells' circumferences traced with an HP 9864A digitizer interfaced with an HP 9830A computer. The measured area was doubled to give total surface area.

Passive properties. R_m was calculated by multiplying R_{in} by the surface area. C_m could then be calculated from the membrane time constant.

Active properties. Voltage, current, and dv/dt records were played back and filmed to measure relevant parameters on the HP minicomputer or they were digitized on a MINC 11/23 computer for similar analysis. Threshold was estimated from AP records as the point which voltage clearly deviated from a completely passive response.

Means are plus or minus standard deviation except as noted. Statistical comparisons were made using the nonparametric Mann-Whitney U-test (Siegel, 1956) which can be used on populations the distributions of which are non-normal or unknown.

Single channel records. These records were analyzed by individual channel analysis and/or summing multiple traces to mimic whole cell currents (Sigworth and Neher, 1980). For both methods, the analogue data was digitized at a 25- to 50-μsec sampling rate on the MINC 11/23.

For summing purposes, 15 to 60 depolarizing pulses were filtered at 3 to 4 kHz (−3 dB frequency; Bessel response) and added along with an equal number of hyperpolarized pulses to remove leakage currents. The resulting peak current was measured by eye. Since, even at 11–12°C, the rising phase of the inward current was not well resolved, only the time-to-peak current and the decay rate of the inward current (τ_h) were routinely measured. τ_h was estimated by fitting the decay current to an exponential by eye. Visual fitting gave τ_h estimates within 8% of theoretical values for synthetic data with superimposed noise. τ_h was corrected to 14°C using a Q_{10} of 2.85 (see Frankenhaeuser and Moore, 1963).

Individual records were filtered at 3 kHz and digitized at 25- to 30-μsec intervals for analysis of single channel currents. Six to ten hyperpolarizing pulses of equal amplitude were averaged, and the average was added to each record for leakage correction. The base line and open state level were fitted by eye. The open and close transitions were then set visually to coincide with the half-maximal value. Events briefer than 150 μsec were excluded from analysis. In cases in which only a few events (<50) were recorded at a given potential, the open times were averaged for comparison between cells of different culture ages. These values overestimate the true mean open time by an amount equal to the short duration cutoff point (i.e., 150 μsec; Neher and Steinbach, 1978). The averaged open times and open times determined by fitting histograms with a single exponential (τ_o) were corrected to a temperature of 14°C using a Q_{10} of 2.3 (Frankenhaeuser and Moore, 1963).

Results

Excitability in muscle cells

The majority of experiments described here were done on cultures from stage 18 to 20 embryos. Results from stage 18 embryos (pre-innervation; Kullberg et al., 1977; Chow and Cohen, 1983) did not appear different from those on older embryos (up to stage 27).

Cultured *Xenopus* myotomal cells remain healthy for more than 1 week under the culture conditions used here. During the initial 3 to 4 days, they grow to their maximum size, approximately doubling their surface area (Table I). Figure 1 shows examples of a small 1-day-old muscle cell and a 5-day-old cell. When examining the cells' electrical properties, there was often considerable variability. To

TABLE I
Properties from sister cultures

Days in Culture	Surface Area (μm^2)[a]	E_r (mV)	R_{in} (megohms)	τ_m (msec)	R_m[b] (kilohm$-cm^2$)	C_m[c] $(\mu F/cm^2)$	$\dot{V}_{max}$ (V/sec)
1	3565 ± 1416 (61)[d]	-98 ± 8 (6)	256 ± 122 (7)	18 ± 5 (7)	9.1	2.0	26 ± 26 (5)
3	7478 ± 3324 (50)	-93 ± 6 (17)	134 ± 43 (17)	17 ± 7 (17)	10.0	1.7	26 ± 28 (14)
6	6964 ± 3011[e] (61)	-96 ± 6 (13)	122 ± 40[e] (14)	17 ± 4 (17)	8.5	2.0	82 ± 40[e] (13)

[a] Values for surface area, E_r, R_{in}, τ_m, and $\dot{V}_{max}$ are expressed as mean $\pm$ SD.
[b] $R_m = R_{in} \times$ surface area.
[c] $C_m = \tau_m/R_m$.
[d] Numbers in parentheses, number of cells.
[e] Statistically significant change between days 1 and 6, $P = 0.001$, Student's t test.
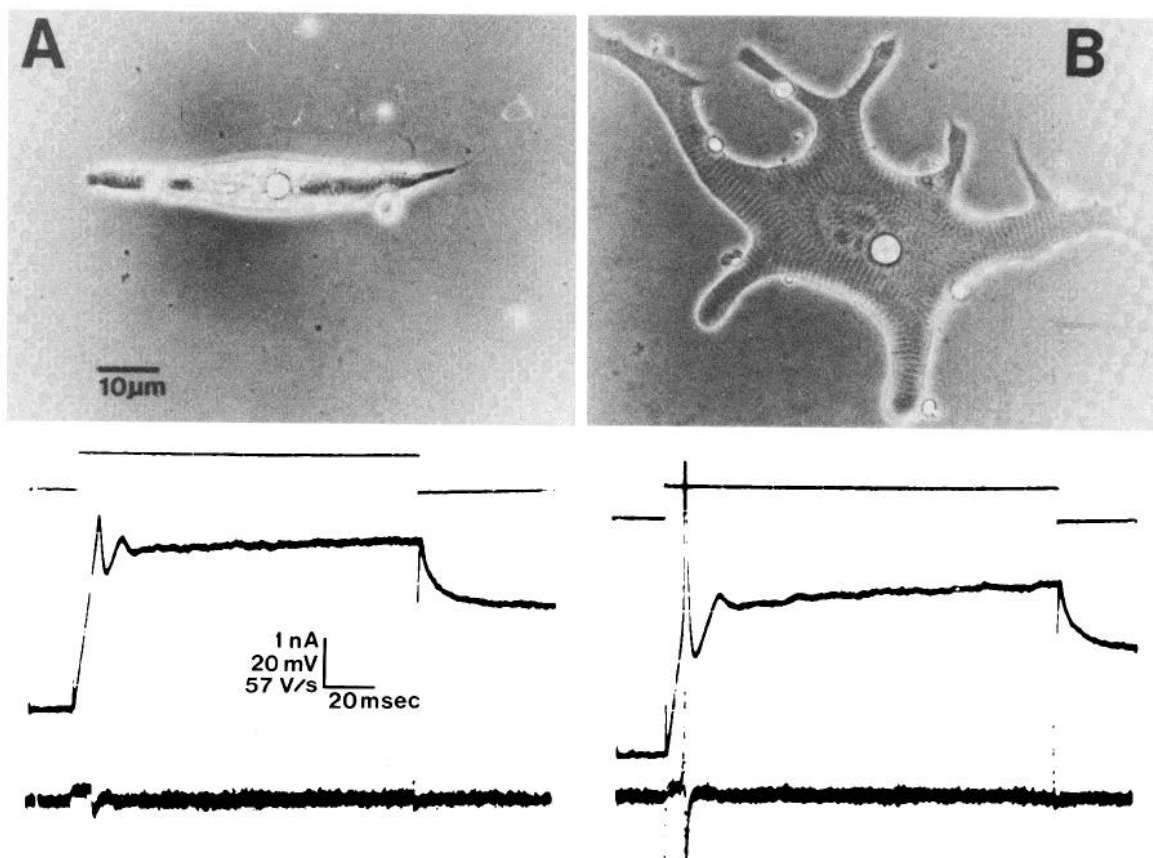

Figure 1. Morphology and action potentials from different aged cells. *A* shows a typically shaped, but somewhat small, 1-day-old muscle cell and a representative action potential from a different 1-day-old cell. *B* shows a 5-day-old muscle cell. In each *lower panel*, the top trace is current, the *middle trace* is voltage, and the *bottom trace* is dV/dt. Zero current also represents the zero potential point. The voltage did not return to the previous resting level after the stimulation ended due to contraction-induced damage.

minimize this variability, cells were compared between sister cultures whenever possible. In addition, although myotomal cells do not fuse in culture, they do become electrically coupled to contacted myotomal cells. Therefore, all intracellular measurements were made on isolated cells.

Passive membrane properties. Table I shows the data on passive electrical properties obtained from one set of sister cultures sampled at 1, 3, and 6 days. The specific membrane resistance ($\sim$10 kilohms-cm^2) and capacitance ($\sim$2 $\mu F/cm^2$) do not change while the input resistance falls to about half its initial value by day 6. The reduction in input resistance can be accounted for entirely by the increase in the cells' surface area. The value for C_m is higher than that found in nerve and most likely reflects a contribution from transverse tubules

as seen in the electron microscope (G. Klier, personal communication). The resting potential is also unchanged, as measured by intracellular methods. This result was later confirmed using the patch electrode (see "Materials and Methods") where 1-day-old muscle cells had a resting potential of -89 ± 3 mV ($n = 7$).

Ionic channels involved in the action potential. The shape of the active response to depolarization, at least in older cultures, suggested that the ion channel involved in the upstroke was the voltage-dependent Na channel (Figs. 1 and 2). Substitution of extracellular Na with Tris completely abolished the spike in 1-day-old and 6-day-old muscle cells. As an additional test, 0.1 to 3 μM tetrodotoxin (TTX) was added to the bathing solution. TTX eliminated the fast spike in young (1-day; Fig. $2A_2$) and older (5-day; Fig. $2B_2$) cells;

Figure 2. Ionic dependence. *A* is from 1-day muscle cells; *B* from 5-day-old cells. A_1 and B_1 are responses from cells in normal recording solution. In A_2, B_2, and B_3 TTX (3 μM) and tetraethylammonium (10 mM) have been added to the bathing solution. A_3 and B_3 are examples of the prolonged depolarization following stimulus cessation. *Top trace,* current; *middle trace,* voltage, *third trace,* if present, dV/dt. *Vertical scale bar* is 0.4 nA, 20 mV, or 114 V/sec. *Horizontal scale bar* is 20 msec in A_1, B_1, A_2; 50 msec in B_2; 100 msec in B_3 and 200 msec in A_3.
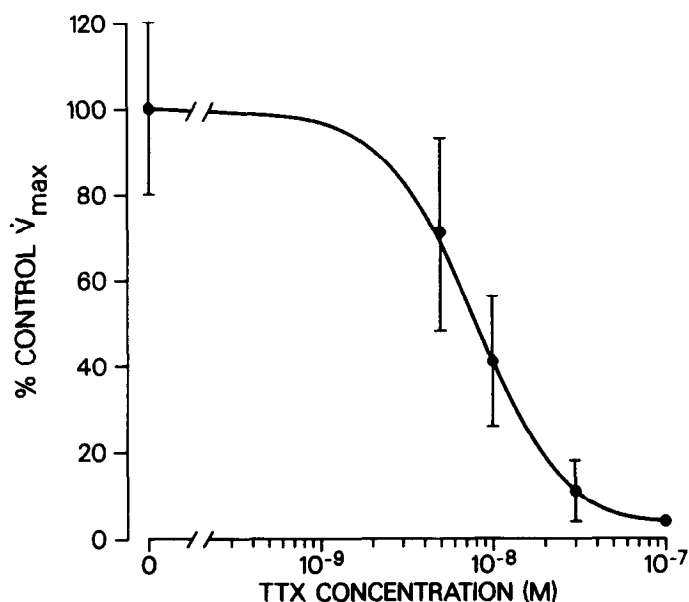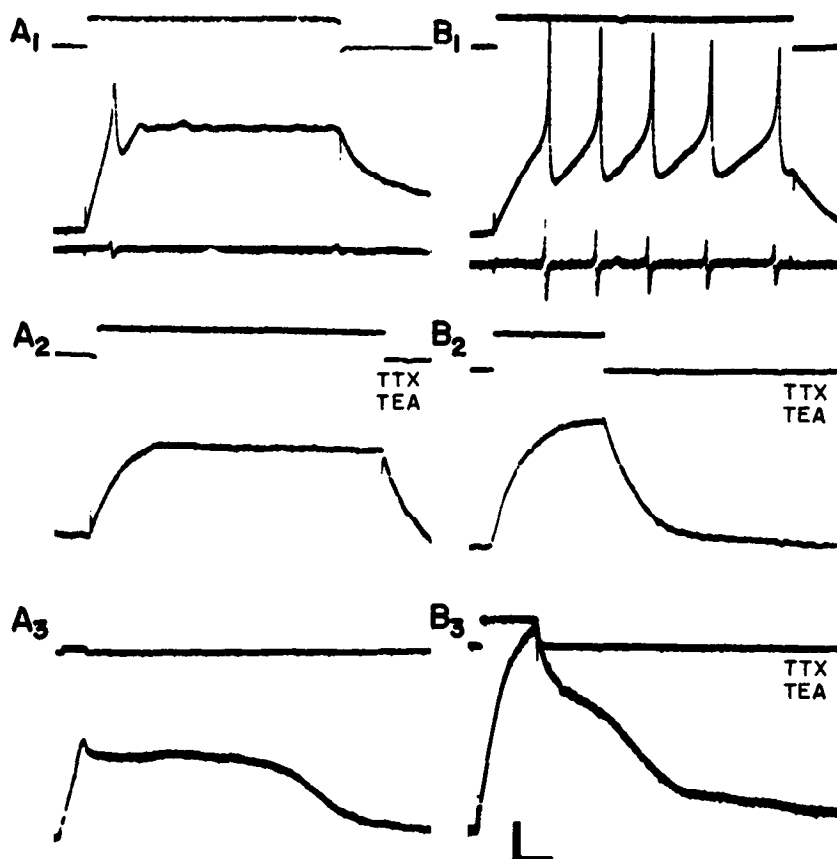

Figure 3. $\dot{V}_{max}$ sensitivity to TTX. Each *point* is the average ± SE of seven to nine points. Control $\dot{V}_{max}$ is the response in 0 TTX. The *curve* is drawn by eye.

Figure 4. Examples of $\dot{V}_{max}$ versus time in culture. Each *point* represents the mean ± SE (n = 8 to 25). Means from sister cultures at different times are connected to one another. O, stage 19 embryos; ▲, stage 22 embryos; ●, stage 19 to 20 embryos.

TTX did not reveal a second inward current during the stimulus (however, see below).

Since immature or denervated muscle cells are often less sensitive to TTX, the TTX concentration was measured which reduced $\dot{V}_{max}$ by half ($K_{0.5}$). This experiment was only performed on cells of at least 3 days in culture, since $\dot{V}_{max}$ was sufficiently small in younger cells to make this measurement inaccurate. Figure 3 shows the dose response curve from cells 3 days after culturing. $K_{0.5}$ does not shift
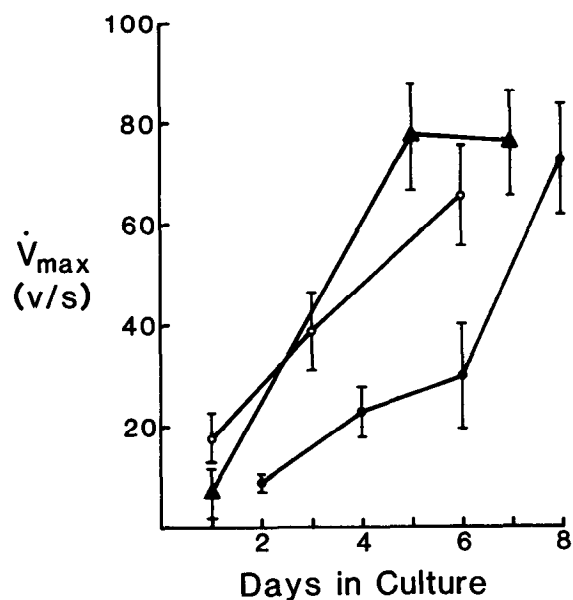
to smaller values in older cells over the time period examined; the mean $K_{0.5}$ for 6 experiments from 3 to 7 days *in vitro* was 9 mM. These results indicate that there is not a significant TTX-insensitive channel population present after 3 days.

Both delayed and anomalous rectification were observed from steady state I-V plots in most cells as early as day 1 (data not shown). No effort was made to characterize these potassium channels. However, the delayed rectifier may obscure an inward Ca
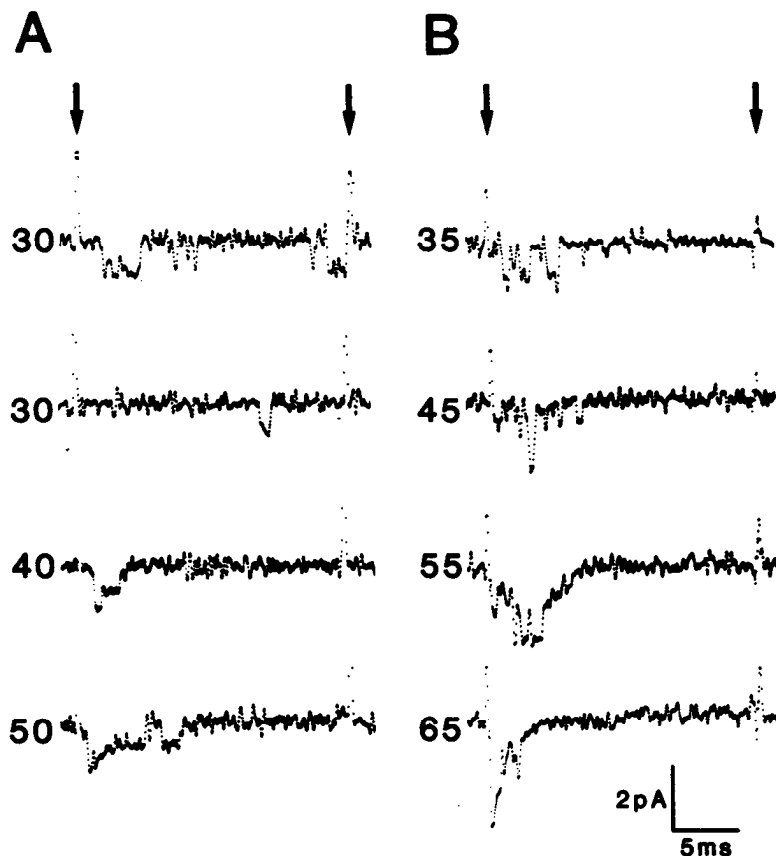
A   **B**

Figure 5. Single channel records from 1-day-old (*A*) and 4-day-old (*B*) muscle cells. *Arrows* indicate on and off time of stimulus pulse. Voltage steps from rest are indicated to the *left* of each record. Sample interval = 30 μsec; f_c = 2 kHz. T = 10°C in *A*, 13°C in *B*.
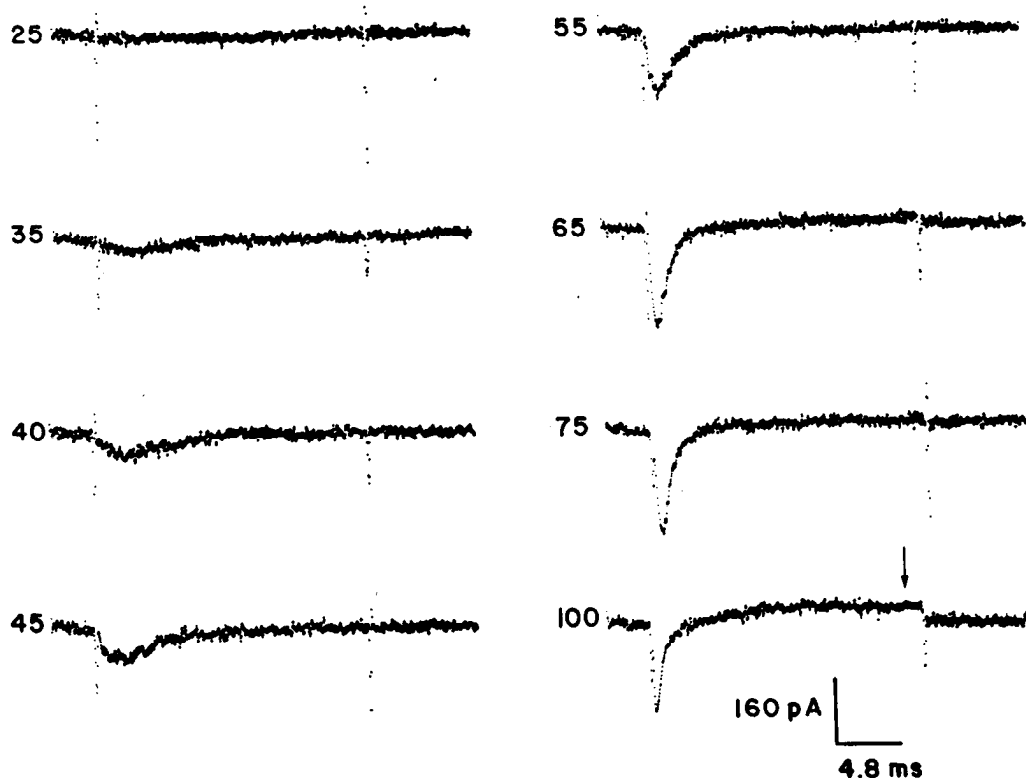
Figure 6. Summed single channel records from a 4-day-old muscle cell. Potential step from E_r is given to the *left* of each trace. Sample interval = 30 μsec; f_c = 4 kHz; T = 13° C. 50 depolarizing records were added at each voltage step. The *arrow* indicates outward current.

current which has been observed in other developing systems. Indeed, on occasion, a slow repolarization was seen after the stimulus was turned off. This slow repolarization occurred in young cells without any channel blockers present (Fig. 2A_3) and in mature cells in the presence of TTX and tetraethylammonium (Fig. 2B_3). To

unmask a possible Ca current, especially during the stimulation period, 10 mM tetraethylammonium and 5 mM 4-aminopyridine were added to the bath along with TTX. However, from steady state I-V plots, these two compounds did not appear to completely block the K conductance when applied to the bath. Bath applied Ba (0.25 to
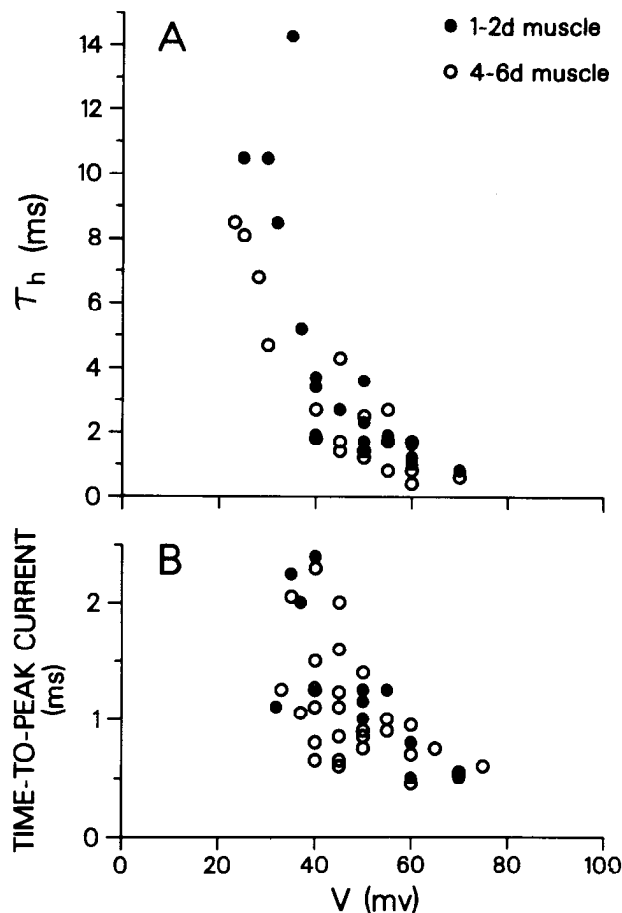
Figure 7. τ_h and time-to-peak current for two age groups. A shows $\tau_{h,}$ and B shows time-to-peak current plotted against voltage: ●, young cells; ○, old cells. These parameters are not corrected for temperature. $T = 14 \pm 1°C$.

Figure 8. Open time histograms. $P(t)$ is the probability that the opening is t msec or longer. A, from 1-day muscle cell; $T = 15°C$, $\tau_o = 0.38$ msec; B, 4-day muscle cell, $T = 13°C$, $\tau_o = 0.44$ msec.

1 mM) was also ineffective in unmasking Ca channels, since the membrane potential was very unstable with this cation present. Since the slow repolarization off response was infrequent and the ability to uncover another possible inward current was unsuccessful, the conductance underlying the off-response was not further studied. Therefore, we cannot rule out the possibility that a Ca component was involved during the steady-state phase of the depolarizing pulse or in the off response. The change in Na current with time was next studied by measuring $\dot{V}_{max}$.

Rate of rise of the action potential. A bathing solution of high Na (120 mM) and Ca (10 mM) was used in these experiments to facilitate detecting Na and Ca components in the AP and to stabilize the membrane potential. In the high Na/Ca solution, action potentials often appeared within 24 hr after culturing of the myocytes. $\dot{V}_{max}$ was measured to study the change in the AP with time. Figure 4 shows the change in $\dot{V}_{max}$ in three different sets of cultures. Often by day 5, $\dot{V}_{max}$ had reached its maximum value of 46 to 141 V/sec, although the cultures were never examined beyond 8 days. In nine sets of cultures made from stage 18 to 20 embryos the average increase in $\dot{V}_{max}$ was 7.5-fold from the first observation (day 1 to 2) to the last observation (≥5 days). Cultures from older embryos (up to stage 27) showed the same trend. In addition to having a faster rate of rise, older cells *in vitro* occasionally displayed multiple APs during a prolonged depolarization (see Figs. 1 and $2B_1$).

The augmentation in $\dot{V}_{max}$ reflects an increase in Na current density, since the specific membrane resistance and capacitance do not change. The possible explanation for this finding is a shift in Na channel activation to more hyperpolarized potentials. This shift would result in more Na channels becoming activated at a given
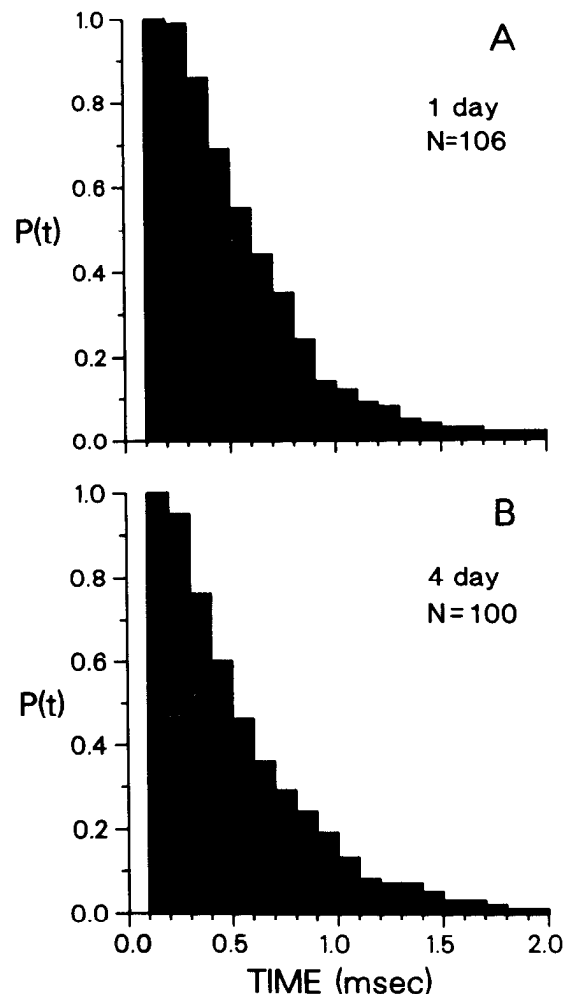
voltage, thus increasing $\dot{V}_{max}$, while reducing the number of channels being inactivated during the time required for the membrane to discharge. To test for a shift in Na channel inactivation, threshold was estimated. Threshold was similar for 1-day-old cells (−36 ± 8 mV; $n = 15$) and 6- to 7-day-old cells (−38 ± 9 mV; $n = 22$). This result and evidence from single channel analysis presented below suggest that no voltage shift in activation kinetics occurs as the cells mature.

Single channel currents. At least three possibilities could account for the increased Na current during the AP: (1) increased single-channel conductance in mature cells; (2) alterations in activation and/or inactivation kinetics; and (3) an increase in Na channel density. The first two possibilities can be examined electrophysiologically. Indirect evidence supports the last possibility.

For these experiments, cultures were separated into two age groups: young cultures were 1 to 2 days old, and old cultures were at least 4 days old. Single-channel recordings were obtained from cell-attached patches to closely mimic the conditions in which $\dot{V}_{max}$ was measured. Although the membrane potential is unknown in this instance, we showed earlier that there were no changes in the cells' membrane potential during development *in vitro*. Therefore, comparisons of single-channel properties between different aged cells is valid.

Initially, single-channel recordings were made using the same solution in the electrode as in the bath with 10 mM Ca. However, individual Na currents were difficult to resolve in high Ca. Therefore, the Ca was removed and EGTA (0.5 mM) was added. This solution
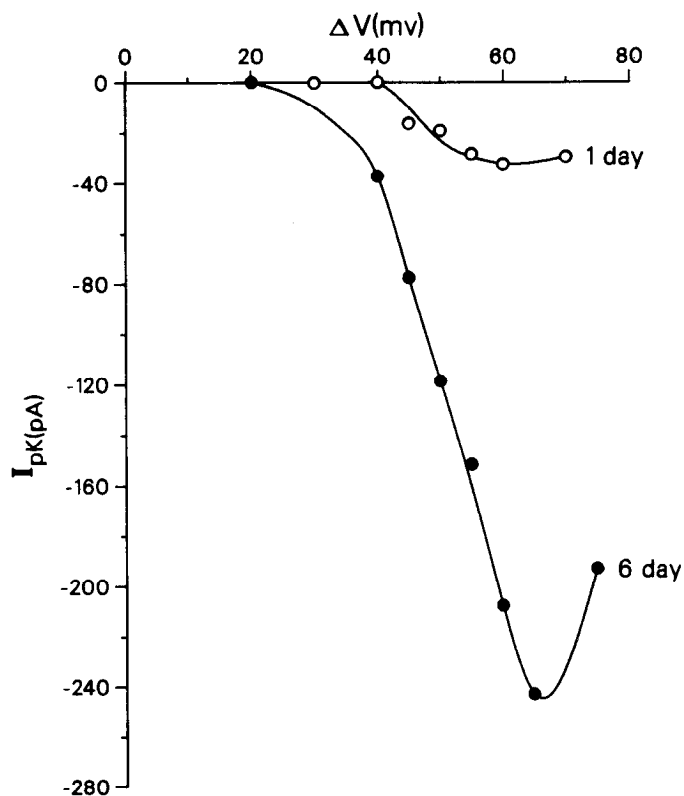
Figure 9. I-V plot of voltage step versus peak inward current obtained from summed records from sister cultures. 50 depolarizing traces at each potential were added. $T = 9°C$ for 1-day cell, 12°C for 6-day cell.

resulted in a two-fold increase in unitary current amplitude. This augmentation in Na current amplitude is likely due to either the decreased surface potential screening (Ohmori and Yoshii, 1977) or the removal of the blocking effect by Ca (Yamamoto et al., 1983) when Ca is absent from the external surface of the membrane.

To test that the single-channel currents we recorded were actually arising from Na channels, either 3 μM TTX was added or Na was removed from the electrode solution (with Tris substitution). In nine patches where this was done, none displayed any single-channel activity. In contrast, 85% (79/93) of the patches from all aged cells (1 to 6 days) with the normal electrode solution had channel openings during a depolarizing voltage step of 50 mV or more.

In six of 20 patches examined closely, there were individual outward current events. In these cases, the presumptive K currents appeared at potentials steps of 50 mV or more from the resting potential. The driving force for K current is not very large in the range of potentials used here, so many of the outward K currents may go undetected. Because they appeared later during the stimulus than Na channels, there was not a problem with contamination, except occasionally when measuring τ_h (see below).

Figure 5 shows examples of single-channel Na currents from a 1-day and 4-day culture, each at a number of different step potential levels. It is apparent that the unitary current does not differ substantially between these different aged cultures. The unitary current for the 1-day culture (Fig. 5A) was 1.7 pA and, for the 4-day culture (Fig. 5B), it was 1.9 pA at 40 mV. The voltage level at which single channel events first appeared was variable, being as low as 10 mV from resting level but more usually starting at 30 mV. This threshold is approximately −65 mV in absolute terms, which is considerably more hyperpolarized than threshold measured intracellularly (−36 to −38 mV). The shift, however, is expected since, in the patch recording experiments, the stabilizing effect of Ca on the membrane has been removed.

After Na currents were first detected, the potential was stepped

in increments of 2 to 10 mV. Because step size varied, the numbers of records at any given potential is small, but comparisons at multiple voltage levels show consistent trends. Comparisons of the unitary currents from seven young (1.7 to 2.7 pA) and seven old cells (1.8 to 2.6 pA) at 40 mV revealed no differences by the nonparametric Mann-Whitney U-test (Siegel, 1956). The single-channel events were composed of a single class of amplitudes although, in a few patches, there appeared to be a channel of smaller conductance. However, its frequency of occurrence was very low, and it was not included in the single channel analysis. Due to low numbers of resolvable single-channel events at each multiple potential steps, unitary conductance (γ) estimates were obtained from the slopes of I-V plots in only a few cases. From five young cells, γ was 24 ± 9 pS; in older cells ($n = 7$), γ was 28 ± 6 pS ($T = 14 ± 2°C$). This difference is not significant (Mann-Whitney U-test: $p > 0.1$).

Since an increase in unitary conductance did not seem to be the mechanism by which these cells increase their Na current, an alteration in channel kinetics might be responsible. Slowing the inactivation process or augmenting activation could result in increased Na current. Channel kinetics were examined by first measuring the decay time constant (τ_h) of current traces obtained by summing a number of sequential single channel records (Sigworth and Neher, 1980). An example of such a treatment is shown in Figure 6, which is from a 4-day-old cultured myocyte. In most cases, the decay of current could be well fit by a single exponential. Where there seemed to be more than one component, the initial, faster portion was fit to a single exponential, and this time constant was used for τ_h. Any contribution by a second, slower component may arise in part from outward K current (see Fig. 6; $V = 65, 75$, and 100 mV). Figure 7A plots τ_h against potential for the two age groups. As can be seen in this figure, the older cells do not have a longer time course of current decay which would prolong Na flux through its voltage-gated channel.

From Figure 6, one sees that the activation step is very fast. By cooling the preparation, we had hoped to better resolve single-channel openings; however, in all but two instances (see for example, Figure 6), the rising phase of the summed response could not be detected clearly, even when cooled to 9°C. This was due in part to incomplete cancellation of the stimulus artifact. It was not possible, therefore, to measure a time constant for the activation process. As an indirect estimate of activation, the time-to-peak current was measured and plotted for each potential (Fig. 7B). As with τ_h, there is no indication that older cells have altered their activation kinetics.

As another means of looking at the kinetic properties of the Na channel, the open time of individual channels can be measured. Averages were used to compare the two age groups, although these values overestimated the true opentimes (see "Materials and Methods"). Comparisons of average opentimes at a given potential level showed no differences. For example, at 40 mV, the average open time was 0.60 msec ($n = 9$) and 0.53 msec ($n = 9$) for young and old cells, respectively. Under optimal conditions, open time histograms were constructed when there were more than 50 events in order to obtain approximate mean open time (τ_o) values. In general, these histograms were well fit by eye to a single exponential in spite of the relatively few numbers of events. The two examples shown in Figure 8 (A, 1-day; B, 4-day) taken at 35 mV are very similar. At 40 mV, τ_o was 0.60 ± 0.12 ($n = 3$) for young cells and 0.45 ± 0.47 ($n = 6$) in old cells. This also supports the conclusion that there is no obvious alteration in the Na channel opentime during in vitro development.

The increase in V_{max} seen with time in culture reflects an increase in inward current during the AP upstroke. To better substantiate this finding that inward current density increases, the peak current (I_{pk}) was measured from summed current records. Maximum I_{pk} was determined only in those cells in which the potential steps were great enough to cause I_{pk} to pass through a maximum value. For young cells, the maximum I_{pk} elicited was in the range of 32 to 162 pA ($n = 4$) for 50 summed records. This does not include two
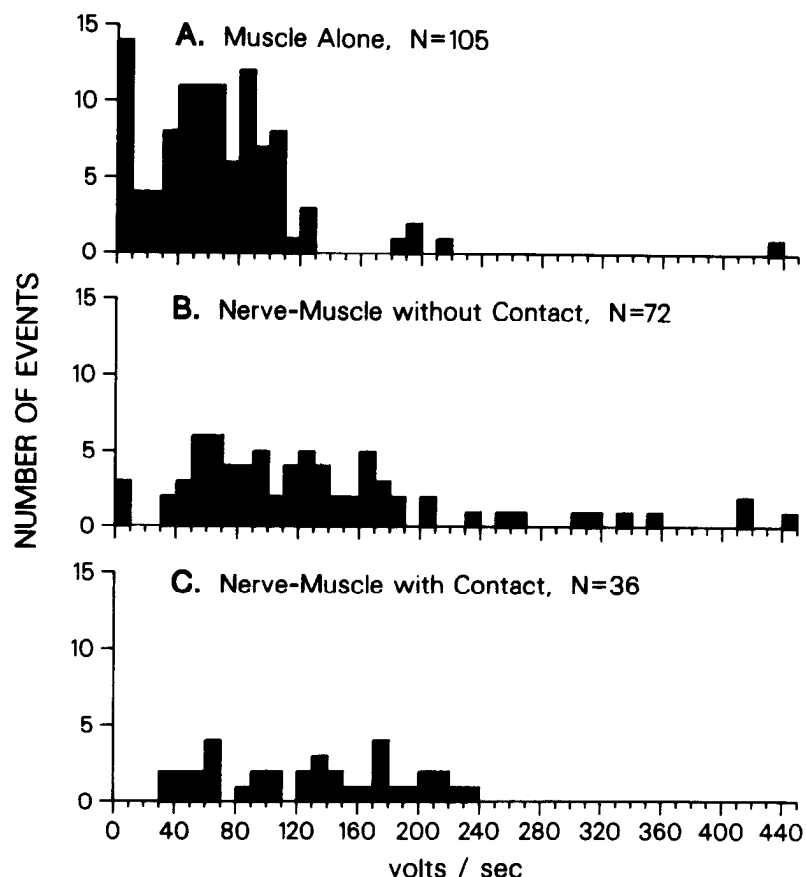
Figure 10. Histograms of $\dot{V}_{max}$. $\dot{V}_{max}$ was measured in cells from 11 muscle cultures (*A*) and 13 nerve-muscle cultures in which the cells were classified either as contacted by neurites (*C*) or not contacted (*B*).

patches which had channels but undetectable summed current (<5 pA for 50 summed depolarizing records). In old cells, the maximum current range was 197 to 338 pA ($n = 4$) for the same number of added records. Statistically, this change in I_{pk} represents a significant difference between young and old cells ($P = 0.014$; Mann-Whitney U-test). The size of the electrode which probably effects the surface area of the patch of membrane being recorded from was similar for all of these cells. The potential at which this maximum current was reached was approximately 60 mV from rest in both cases. Figure 9 shows a particularly good example in which I_{pk} was compared between two sister cultures at 1 and 6 days *in vitro*. Here the maximum I_{pk} is 7 times greater at 6 days than at 1 day. It should be noted that the apparent shift in threshold for inward current at 1 day (Figure 9) is due to the inability to detect small summed currents. In fact, single channel events were present at 30 and 40 mV in the 1-day-old cell. The above results raise the possibility that since unitary conductance and channel kinetics do not change developmentally, then channel density most likely is increasing.

The evidence presented suggests that cultured *Xenopus* muscle cells augment their inward current during an AP by increasing Na channel density. In support of this hypothesis, the percentage of membrane patches containing Na channels (determined by visually monitoring at least 20 stimuli at depolarizations at 50 mV or more) was 75% (39/52) in young cells and 98% (40/41) in older cells. Even in patches containing Na channels, I_{pk} for summed records was undetectable in two of 11 young cells, indicating that there were too few channel openings to make a significant contribution. On the other hand, in older cells all 17 of 17 summed records had measurable I_{pk}.

Effects of neural tube addition on muscle excitability

Maximum rate of rise of the action potential. Nerve-muscle co-cultures were examined 2 days or more after the addition of neural

tube cells. The muscle cultures were at least 4 days old, so the Na-dependent AP was already well developed in these cells. $\dot{V}_{max}$ was compared between muscle cultures with and without neural tube. In addition, cells in the cocultures were identified as to whether they were nerve-contacted. Figure 10 shows histograms of $\dot{V}_{max}$ values obtained from cells in muscle cultures (Fig. 10*A*), nerve-contacted cells (Fig. 10*B*), and noncontacted cells in cocultures (Fig. 10*C*). As can be seen, there is wide variability in each group. Much of the dispersion arises from comparing nonsister cultures, but there was considerable variation even between cells within a given culture dish or between different sister culture dishes. $\dot{V}_{max}$ from cells in coculture, whether neurite-contacted or not, showed a significant increase when compared to $\dot{V}_{max}$ from muscle cultures ($p < 0.001$). The overall increase in $\dot{V}_{max}$ is 2.3-fold when cells from panels *B* and *C* are combined. It thus appears that actual nerve contact is not a prerequisite for increased $\dot{V}_{max}$, although the classified "noncontacted" cells in coculture may at some time before recording have been in contact with a neuron. For the remainder of the paper, all cells in nerve-muscle coculture are considered together and as a group are compared to cells in muscle cultures.

The cause for the increase in $\dot{V}_{max}$ was not due to changes in resting potential or threshold. The average resting potential in co-cultures was the same (-92 ± 6 mV; $n = 59$) as in muscle cultures (-92 ± 6 m V; $n = 77$). Threshold measured in two experiments was comparable between sister muscle and nerve-muscle cultures (-42 versus -40 mV and -45 versus 50 mV). Therefore, the augmented $\dot{V}_{max}$ suggests that neural tube addition causes muscle cells to increase their Na current density. Subsequent single-channel experiments on muscle and nerve-muscle cultures were performed to determine the mechanism for the increased $\dot{V}_{max}$.

Single-channel currents. The previous results suggested the increase in $\dot{V}_{max}$ seen in cocultures could be accounted for by an increase in Na channel density without alterations in individual Na

A   B

158pA
5pA
5ms
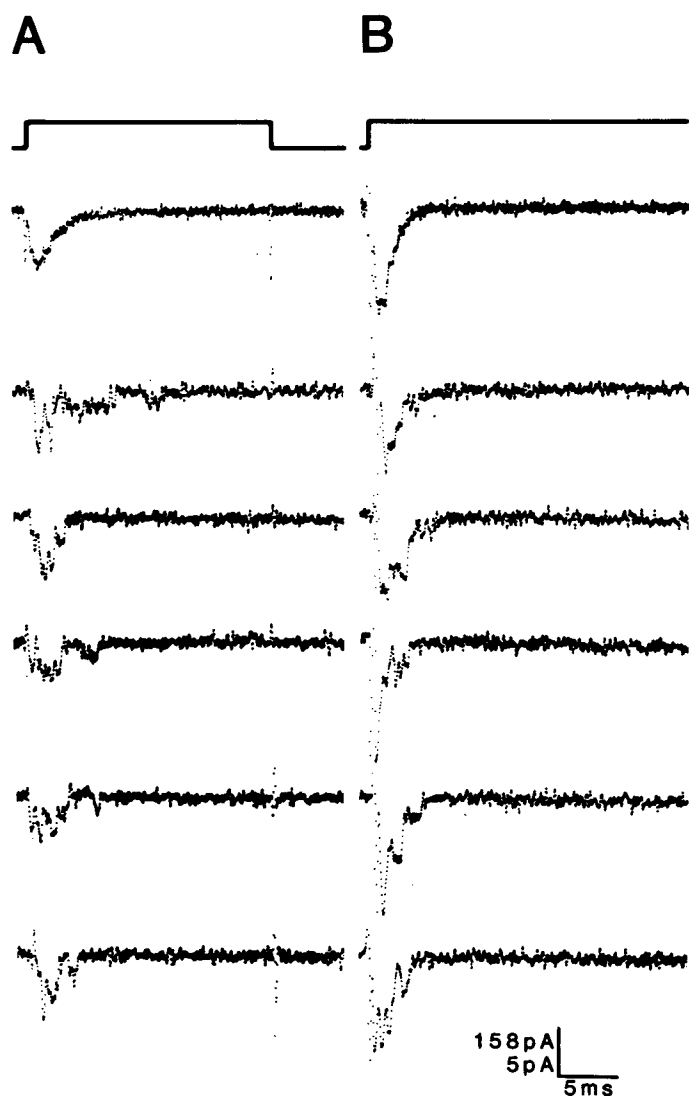
Figure 11. Examples of single channel records and summed currents from cells in muscle cultures (*A*) and nerve-muscle culture (*B*). The *top trace* is the 55 mV voltage step. The second record is the summation of 50 individual records, examples of which are shown in the last five traces of each panel. The Bessel filter was set at 4 kHz (−3 dB point) for the summed record and 3 kHz for the last five records. Sample interval is 30 μsec. *Top number* on the *vertical scale bar* is for summed currents; the *lower number* is for individual records.

channel parameters. We, therefore, examined single-channel records to determine if the same mechanism applied for increasing Na current in the nerve muscle cocultures.

The two types of records obtained from cell-attached patches are shown in Figure 11 for a muscle culture (Fig. 11*A*) and a nerve-muscle coculture (Fig. 11*B*). The *top trace* is the depolarizing voltage step. The last five records in each panel are examples of single-channel current records in response to a 55-mV depolarizing pulse. The second trace is the summed current record from 50 such responses after correction for leakage current. I_{pk}, the decay time constant and time-to-peak current, were compared between the two types of culture. Of 34 and 37 patches examined from muscle and nerve-muscle cultures, respectively, all showed channel activity. In five cases, when 3 μM TTX was present in the electrode, no channel activity could be detected on cells in nerve-muscle cultures, as was the case for muscle cultured alone (see above).

To verify that the increase in V_{max} reflects an increase in Na current density, I_{pk} was measured. However, at both 40 and 50 mV, I_{pk} was
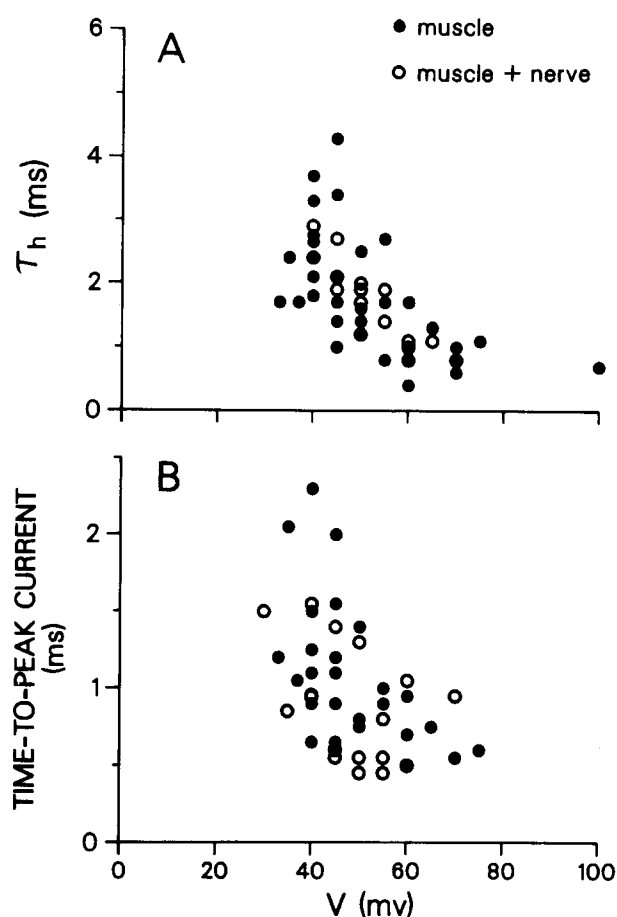
Figure 12. τ_h and time-to-peak current as a function of voltage. Values from muscle cultures (●) and nerve-muscle (○) overlap in some cases (*slightly larger filled circles*) $T = 14 \pm 1°C$.
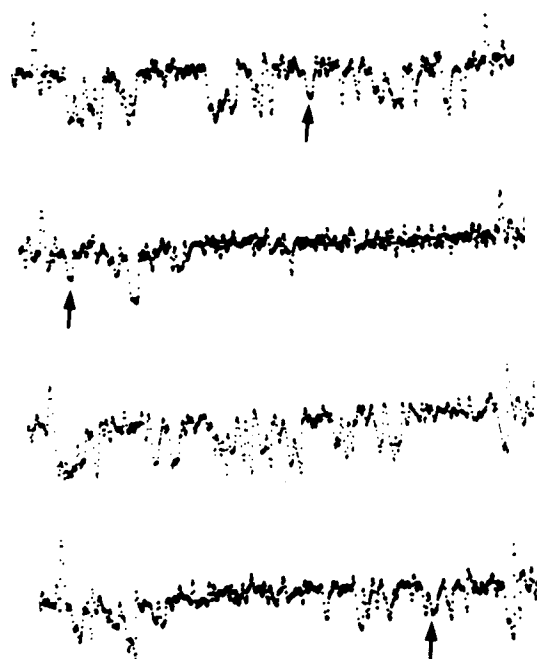
Figure 13. Example of single channel current records from a 4-day nerve-muscle culture. Neural tube was added 3 days previous to recording. All records were in response to a 40-mV depolarizing pulse. *Arrows* show examples of the smaller current amplitude channels.

Figure 14. Amplitude (*left panel*) and open time histograms for muscle (*A*) and nerve-muscle (*B*) cultures in response to a 40-mV step. Open time histograms (*right* panel) are plotted as $P(t)$ versus t where $P(t)$ is the probability that the measured value is greater than t. The mean open time (τ_o) was calculated by the best visual fit of a single exponential. $\tau_o = 0.14$ msec in A; $\tau_o = 0.26$ msec for B. It should be noted that the mean current amplitude for the muscle cell in A is smaller than normal.
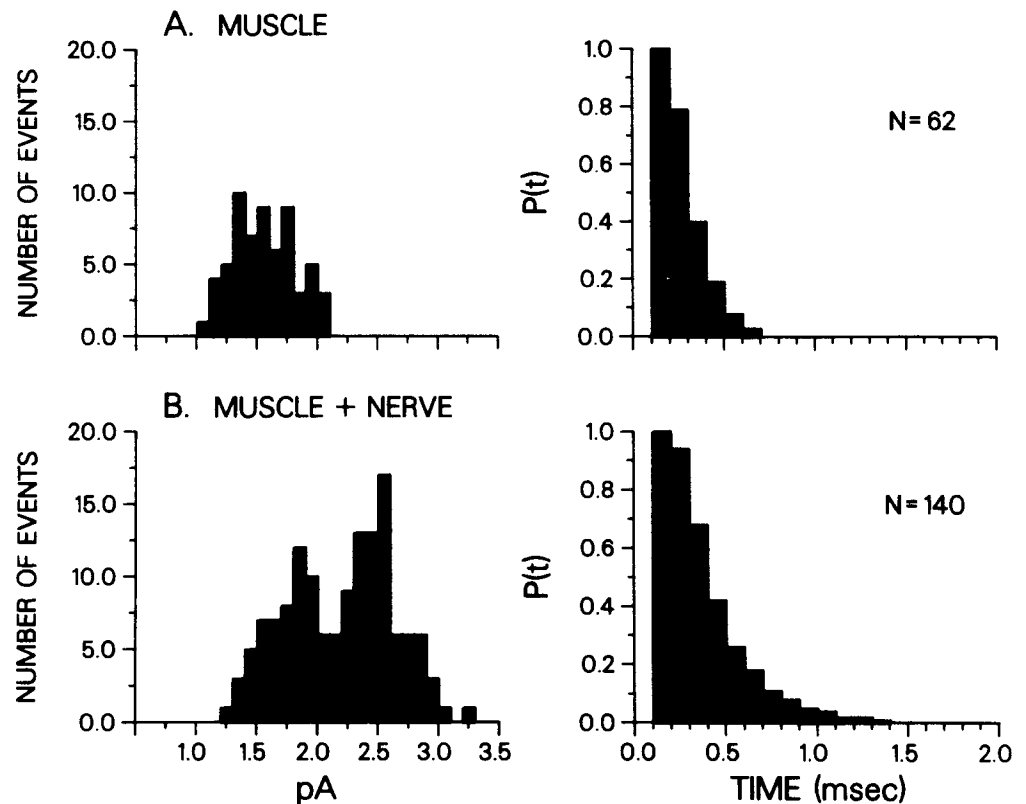
not significantly larger in nerve-muscle cultures compared to muscle cultures ($p > 0.1$, $n = 6$ to 12) in spite of the apparent augmented range for cells in coculture: the range of I_{pk} at 40 mV was 0 to 211 pA for muscle cultures and 0 to 427 pA for cocultures; at 50 mV, these respective ranges were 49 to 201 pA and 38 to 1003 pA. The apparent discrepancy between V_{max} and I_{pk} measurements may be due to the small increase (two-fold) in V_{max}, which is not large enough to be detected with I_{pk}. We continued to characterize individual Na channels to ascertain the influence of neural tube addition on channel kinetics and conductance.

The time course of the summed current records was examined to determine whether activation and inactivation properties were affected by the addition of neural tube. In most cases, the time course of current decay was well fit to a single exponential. The values of τ_h at different potential steps for five muscle cultures and seven nerve-muscle cocultures are shown in Figure 12*A*. τ_h becomes smaller as the membrane patch is depolarized. In nerve-muscle cocultures, τ_h is similar over a wide range of potentials compared to τ_h from muscle cultures. Time-to-peak current values are plotted against potential for both culture types in Figure 12*B*. Eight cells from muscle cultures and four cells from nerve-muscle cultures are included in this figure. As with τ_h, time-to-peak current is not altered after the addition of neural tube cells to muscle cultures. These similarities suggest that the activation and inactivation processes underlying the time course of the summed currents are insensitive to neural tube addition.

Open times and unitary currents were measured from 12 cells in nerve-muscle cocultures. In three cases, only one class of Na channels was present. These channels were 2.4 pA in amplitude at 40 mV depolarized from resting potential and had a unitary conductance (γ) of 28 pS. This is the same conductance value as seen in comparably aged muscle cultures (see above). In the majority of cases (9/12), the single-channel currents appeared to be composed of at least two conductance states (see Figure 13). However, amplitude histograms usually failed to reveal multiple discrete peaks, mainly due to the proximity of the two amplitude classes (the small

class being about 70% of the large amplitude class) and the small number of events at any given potential. In one case, shown in Figure 14*B*, two peaks were discernible. The calculated unitary conductance from these cells ranged from 12 to 29 pS. However, the unitary current-voltage plots were not usually linear in these cases and thus, unitary conductance calculations are inaccurate. This problem is likely due to the presence of the smaller amplitude channels. Since individual amplitude classes could not be easily separated, the amplitude coefficient of variance was calculated as an alternative means of determining the existence of two or more amplitude sizes. However, for cells in coculture ($n = 12$), as well as in muscle cultures ($n = 11$), the coefficient of variance was 0.17 over a 15 to 75 mV depolarizing step range. This finding suggests that while there often appears to be a second conductance class of Na channels in nerve-muscle cultures, the smaller amplitude channels do not make a significant contribution to the total number of recorded single channel events. In fact, the range of amplitude values at 40 mV is not statistically different between muscle and nerve-muscle cultures (1.4 to 2.6, $n = 12$, versus 1.6 to 2.7, $n = 7$, respectively).

While separation of channel types was not done, visual inspection of individual channel openings suggested that smaller amplitude events had similar open times as the larger events. Open time histograms could be well fit to a single exponential for both muscle cultures (Fig. 14*A*) and nerve-muscle cultures (Fig. 14*B*). The averaged open time at 40 mV was 0.45 ± 0.13 msec ($n = 8$) and 0.56 ± 0.13 msec ($n = 8$) for muscle cultures and nerve-muscle cocultures, respectively. At 50 mV, these values were 0.50 msec (± 0.13, $n = 5$) for muscle and 0.46 msec (± 0.09, $n = 7$) for cocultures. The averaged open times show no strong voltage dependence or differences between the two types of cultures.

Discussion

This paper examined the excitability of *Xenopus* myocytes, first developmentally over a 7-day period in culture, and then following the addition of neural tube. In muscle cells grown alone, the Na-

dependent AP undergoes a 7.5-fold increase in $\dot{V}_{max}$. While $\dot{V}_{max}$ cannot be considered directly proportional to membrane current due to lack of isopotentiality during the AP, the observed increase in $\dot{V}_{max}$ undoubtedly reflects a substantial increase in Na current density.

There is little evidence to suggest that the increased inward current is due to a shift in ionic dependence from Ca to Na, although occasionally there is a prolonged depolarization after the stimulus is turned off which might indicate the presence of a Ca component. This contrasts with other muscle systems *in vitro* (Kano et al., 1972; Kidokoro, 1975; Spector and Prives, 1977), where there is an obvious early developmental dependency on Ca. Perhaps a major Ca component disappears before the earliest time the cells are cultured (stage 18) or during the initial hours in culture prior to the first observations (>20 hr). Delayed and anomalous rectification were present from the first day in culture.

Three mechanisms were examined which could account for the increased Na current using single-channel analysis: (*1*) greater unitary conductance in older cells; (*2*) alterations in activation and/ or inactivation parameters which might prolong Na flux; and (*3*) increased Na channel density. Unitary current and conductance (24 pS and 28 pS at 1 to 2 days and 4 to 6 days, respectively) did not increase significantly. The unitary conductance of 24 to 28 pS is higher than reported in rat myoballs ($\gamma = 18$ pS; Sigworth and Neher; 1980), which is probably due to the ionic composition in the patch pipette. With relatively short open times (0.4 to 0.6 msec) at moderate potential steps (less than 50 mV) compared to τ_h (>1.0 msec), the time course of decay reflects slow components of activation rather than inactivation (see Aldrich et al., 1983). So while we were unable to measure directly a time constant for activation because of the rapid onset of channel openings, the slower component, τ_h, did not change, nor did the rapid phase as estimated from time-to-peak current. Likewise, inactivation, as measured by open time, was not altered during the time in culture. Therefore, both conductance and kinetic measurements of individual Na channels show no change during development.

Without actually measuring Na channel density, the evidence suggests that the density of functional channels did increase over time. First, the percentage of membrane patches containing channels increased from 75 to 98%. Second, the maximum I_{pk} was smaller in younger cells, while unitary current remained constant.

In chick muscle, Na channel density was also shown to increase by using a variety of biochemical and electrophysiological techniques (Frelin et al., 1981; Baumgold et al., 1983a, b; Strichartz et al., 1983). Baumgold et al. (1983a, b) further suggested that there are two forms of Na channels: one which binds only scorpion toxin (ScTX) and is nonfunctional and a second, functional type which binds both ScTX and saxitoxin (STX). The density of both binding sites increased over time, with ScTx preceding STX by 1 to 2 days. They suggested that initially nonfunctioning channels which bind only ScTX. Later, the channels become functional and capable of binding both STX and ScTX. Presumably, the immature ScTX-binding (TTX-insensitive) channels all become functional and capable of binding STX (TTX-sensitive) simultaneously so that there is no population of TTX-insensitive, functional Na channels in cultured chick muscle (Kano et al., 1972) as is observed in denervated adult chicken muscle *in vivo* (Harris et al., 1973).

In vivo and *in vitro* rat muscle (Sherman and Catterall, 1982, 1983) also show increases in the density of STX-binding sites. Cultured rat muscle, however, also has a population of TTX-resistant Na channels (Kidokoro, 1975; Sherman and Catterall, 1983) which are functional (Harris and Marshall, 1973; Kidokoro, 1975). In the present electrophysiological study, no effort was made to detect possible nonfunctional Na channels biochemically. However, more than 95% of the Na currents in 3-day and older cultured cells (TTX $K_{0.5} = 9$ mM) were blocked with 0.1 μM TTX, suggesting a negligible, if any, population of TTX-insensitive channels. This is not surprising, since Na channels

on denervated adult frog muscle also are TTX sensitive (Nasledov and Thesleff, 1974).

In the second part of this study, we showed that neural tube addition to muscle cultures led to a further increase in the $\dot{V}_{max}$ of TTX-sensitive Na APs in myocytes, suggestive of an increase in Na current density as observed developmentally. Our results from single-channel analyses can be summarized as follows: (*1*) Time-to-peak current and the time constant for the current decay from summed records were similar in both muscle and nerve-muscle cocultures. In addition, there was no apparent alteration in the voltage sensitivity of these two parameters. (*2*) When only one class of channels was present, the single-channel current and unitary conductance (28 pS) was the same for both culture types. (*3*) The open time was similar in both cases. On the basis of these findings and the observed increase in $\dot{V}_{max}$, we conclude that the addition of neural tube to muscle cultures results in an increase in Na channel density without alterations in the functional parameters of preexisting Na channels.

Interestingly, we found that in most cells (9/12) at least one additional, smaller-amplitude Na channel was present (see Fig. 14*B*). In these cases, the conductance values generally were lower than 28 pS due to contamination by the smaller conductance channels. The smaller amplitude channels likely contributed to the mean current amplitude more at certain voltages than others, making accurate conductance calculations difficult. However, since the different amplitude classes were inseparable, we were unable to determine conductance values for the two classes of Na channels. Instead, we assume that the larger-amplitude class corresponds to the 28 pS channel type; the new, smaller-amplitude channel type has an undetermined unitary conductance. Since the newly appearing channel type does not alter the amplitude coefficient of variance or the range in amplitude, the increased inward current accompanying neural tube addition likely reflects an increased density in the 28 pS channel.

Kano et al. (1979) studied the effect of nerve on chick muscle in a different manner. $\dot{V}_{max}$ measurements were made after nerve extract was added to cultured muscle. The nerve extract addition resulted in an increase in $\dot{V}_{max}$. Since, in the present study, the augmented $\dot{V}_{max}$ was seen in cells whether contacted with neurites or not, the increase in $\dot{V}_{max}$ also may be mediated by a factor released by the neural tube cells. As was pointed out, however, we cannot be certain that the "noncontacted" muscle cells were not, at some point previous to recording, in contact with neurites, which was necessary for increasing $\dot{V}_{max}$. In fact, since neural tubes contain a variety of cell types, even if the enhanced $\dot{V}_{max}$ depends on a factor, such a factor may originate from a non-nerve cell population.

Analysis of our summed current records revealed that the overall time course was unaffected by neural tube addition. As mentioned previously, since τ_h is likely a measure of the activation process (Aldrich et al., 1983), activation and inactivation (reflected by open time measurments) in *Xenopus* muscle are not altered upon neural tube addition. Pappone (1980) showed that denervation of rat muscle also had no effect on τ_h and τ_m (activation time constant).

One of the most interesting results from this study is that Na channels and AChR channels appear to be regulated differently. In the same *Xenopus* muscle preparation, AChRs exhibit a reduction in mean open time as measured by spectral analysis of voltage fluctuations (Brehm et al., 1982) without any alteration in channel density during development (Kidokoro and Gruener, 1982; Brehm et al., 1983a). These and subsequent single channel experiments (Brehm et al., 1983b) showed that the decrease in open time was due to a shift in the relative amounts of two types of AChR channels: one with a mean open time of 0.7 msec and conductance of 60 pS; the other with a 3-msec open time and a conductance of 42 pS. For Na channels, the increase in Na current can be accounted for by an increase in channel density without concomitant functional changes. In the case of AChRs, the cell has effectively increased current through the receptor channel without altering channel den-

sity. This may occur by modification of a single pool of AChRs which ultimately generates different functional AChRs. Alternatively, one might view this as a somewhat similar regulatory mechanism as for the Na channel if the two receptor types originate as two different gene products and only their relative amounts are modified (i.e., changing the density of each).

In the case of muscle cells grown in the presence of neural tube, the smaller-amplitude, longer-open time AChR channels increase in density. For Na channels, neural tube additions appears to induce a second channel type. In addition, the density of the original channel type may increase. Regulation of the Na channel and AChR may occur similarly via modification in the relative expression of two different gene products. In the case of the Na channel, the smaller conducting channel may be expressed in very low levels in muscle cultures alone and go undetected. Alternatively, perhaps the effect of nerve addition is to induce a new type of Na channel. In both cases, developmentally and under neural control, it will be interesting to establish whether the cell regulates Na and AChR channels by similar mechanisms.

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