DEVELOPMENT OF SODIUM CHANNELS DURING DIFFERENTIATION OF CHICK SKELETAL MUSCLE IN CULTURE

II. $^{22}\text{Na}^+$ Uptake and Electrophysiological Studies¹

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Abstract

The development of the functional properties of the sodium channel of chick skeletal muscle grown in culture was studied using ^{22}Na uptake and electrophysiological techniques. In accord with the biochemical data in the preceding paper (Baumgold, J., J. B. Parent, and I. Spector (1983) *J. Neurosci.* 3: 995-1003), the functional manifestations of sodium channel expression are initiated shortly after cell fusion. At this stage, the myotubes have barely detectable sodium-dependent action potentials ($\dot{V}_{\text{max}} = 1$ to 9 V/sec) and exhibit a very small amount of batrachotoxin (BTX)-stimulated $^{22}\text{Na}^+$ uptake. However, when these cultures are treated with scorpion toxin (ScTX), the amplitude and rate of rise of the sodium action potential increase dramatically ($\dot{V}_{\text{max}} = 35$ to 50 V/sec) and the (BTX)-stimulated $^{22}\text{Na}^+$ uptake is much larger, suggesting that ScTX unmasks channels that are already present but nonfunctional in these immature myotubes. The two different rates of development of the biochemical properties of the sodium channel described in the preceding paper are also reflected in the two separate rates of development of its functional properties. In the absence of ScTX, the amplitude and $\dot{V}_{\text{max}}$ of the action potential develop at a slow rate similar to that of the [^3H]saxitoxin binding, eventually reaching a $\dot{V}_{\text{max}}$ of 158 V/sec by day 10; the BTX-stimulated $^{22}\text{Na}^+$ uptake also rises gradually, reaching 12 nmol of $^{22}\text{Na}^+$ /culture/min by day 7.5. In contrast, in the presence of ScTX, the $\dot{V}_{\text{max}}$ of the Na^+ action potential increases more rapidly, reaching 158 V/sec by day 5 and 220 V/sec by day 10. The BTX-stimulated $^{22}\text{Na}^+$ uptake also increases more rapidly in the presence of ScTX. This rapid rate of development is very similar to that for [^{125}I]ScTX binding. These findings and those in the preceding paper suggest the existence of two types of Na^+ channels: an immature, nonfunctional channel capable of binding [^{125}I]ScTX alone, and a mature, functional channel capable of binding both [^{125}I]ScTX and [^3H]saxitoxin. They further suggest that the insertion of the immature form of the channel protein into the cell membrane shortly after cell fusion is the first event in the expression of the sodium channel. During development, the sodium channel undergoes a structural change which renders it functional. The possibility that both the appearance of functional sodium channels during development and the rapid induction of functional channels by ScTX in immature myotubes reflect a post-translational modification or aggregation of immature nonfunctional channels is discussed.

The preceding paper (Baumgold et al., 1983) described the appearance of two distinct binding components of sodium channels from chick skeletal muscle grown in culture. That work demonstrated that during the course

of development of newly fused myotubes, [^{125}I]scorpion toxin ([^{125}I]ScTX)-binding sites appeared first and developed faster than the [^3H]saxitoxin ([^3H]STX)-binding sites. In the present paper we describe the development of the functional properties of the sodium channel and

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the physiological consequences of ScTX binding using ^{22}Na uptake and electrophysiological measurements. We found that sodium channels develop after fusion with two distinguishable rates: a fast rate observed in muscle cultures treated with ScTX and a slower rate observed in untreated muscle cultures. Based on these results, and those described in the preceding paper, we propose a model for the development of sodium channels and for the effect of ScTX on these channels. Parts of this work have been presented in preliminary form (Baumgold et al., 1981; Parent et al., 1981).

Materials and Methods

^{22}Na uptake studies. The muscle cells were grown in 35-mm tissue culture dishes as described in the preceding paper (Baumgold et al., 1983). The ^{22}Na uptake studies were carried out essentially as described by Catterall (1976). Thus, the growth medium was aspirated and the cells were briefly rinsed with toxin incubation medium (130 mM choline chloride, 50 mM HEPES-Tris, pH 7.4, 5.5 mM glucose, 0.8 mM MgSO_4 , 5.4 mM KCl, 1 mg/ml of bovine serum albumin (BSA)), then incubated for 30 min at 37°C with the same medium containing the indicated toxins. This medium was then replaced with a sodium uptake medium containing 120 mM choline chloride, 10 mM NaCl, 50 mM HEPES-Tris, pH 7.4, 5.5 mM glucose, 0.8 mM MgSO_4 , 5.4 mM KCl, 5 mM ouabain, and 1.3 μCi of ^{22}Na (New England Nuclear) per ml. After a 1-min incubation at room temperature, the cells were washed five times (total washing time was less than 10 sec) with 2.5 ml of wash medium containing 163 mM choline chloride, 5 mM HEPES-Tris, pH 7.4, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 1 mg/ml of BSA. The cells were harvested

in 0.4 N NaOH and counted in a Beckman 4000 gamma counter at a counting efficiency of 85%.

Electrophysiology. The electrophysiological experiments were performed on cultures maintained at 35 to 38°C with techniques similar to those previously described (Spector and Prives, 1977). During the experiments, the cells were bathed in a HEPES-buffered (pH 7.3) modification of Eagle's medium (Flow Laboratories, Inc.) which had the following ionic composition: 116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 1 mM NaH_2PO_4 . Cells were impaled by a single microelectrode filled with 3 M KCl (resistance 10 to 20 megohms), which was used for both intracellular current injection and voltage recording.

Results

Development of neurotoxin-stimulated ^{22}Na uptake during muscle differentiation. Mature myotubes have been shown to accumulate $^{22}\text{Na}^+$ in a linear fashion for several minutes in response to stimulation by alkaloid neurotoxins such as batrachotoxin (BTX) or veratridine (Frelin et al., 1981). Thus, the amount of $^{22}\text{Na}^+$ taken up in 1 min is a good measure of the initial rate of $^{22}\text{Na}^+$ uptake in these cultures. In order to determine the development of BTX sensitivity during muscle differentiation, we measured the initial rate of $^{22}\text{Na}^+$ uptake in BTX-stimulated cultures of chick skeletal muscle of various ages. As shown in Figure 1, the initial rate of $^{22}\text{Na}^+$ uptake in 2.5-day-old cultures in response to BTX (200 nM) was 1.3 nmol of ^{22}Na /min/culture, only slightly higher than that of control cultures. By the following day, however, cultures exposed to BTX had an initial rate of $^{22}\text{Na}^+$ uptake double that of control cultures and

Figure 1. Effect of various neurotoxins on the ^{22}Na uptake of muscle cells grown in culture for various lengths of time. The cells were plated at an initial density of 4.2×10^4 cells/cm² as described under "Materials and Methods." The uptake studies were performed, as described under "Materials and Methods," using 300 nM scorpion toxin (ScTX) and 200 nM batrachotoxin (BTX). The background radiation has been subtracted from all values. C represents the amount of $^{22}\text{Na}^+$ taken up in the absence of any neurotoxin. The values shown are the average of three determinations and the error bars represent the standard deviation.

accumulated about 4.5 nmol of $^{22}\text{Na}^+$ /min/culture. This BTX sensitivity gradually increased further during development and reached a maximum of approximately 12.5 nmol of $^{22}\text{Na}^+$ /culture/min by day 7.5. Control cultures, in contrast, showed only a slight increase in their initial rate of $^{22}\text{Na}^+$ uptake, reaching a value of about 3.5 nmol of $^{22}\text{Na}^+$ /culture/min by day 7.5. This gradual increase in the initial rate of $^{22}\text{Na}^+$ uptake in response to BTX stimulation is shown by the *open circles* in Figure 2. This figure demonstrates that as the cultures mature, their initial rates of $^{22}\text{Na}^+$ uptake increase linearly with time between days 2.5 and 7.5 in culture, suggesting that BTX-sensitive sodium channels first appear in 2.5-day-old cultures and increase in number over the next 5 days.

Similar experiments designed to assess the ScTX sensitivity of these cultures did not reveal any increase in the initial rate of $^{22}\text{Na}^+$ uptake above control levels between 2.5 and 7.5 days in culture (Fig. 1), suggesting that under the conditions of these experiments, ScTX alone (300 nM) was unable to cause steady influx of Na^+ ions. However, in the presence of BTX (200 nM), ScTX increased the initial rate of $^{22}\text{Na}^+$ uptake to a value that was considerably higher than that for BTX alone. This cooperative interaction between BTX and ScTX has been described previously (Catterall, 1980) and was already demonstrable in 2.5-day-old cultures, which exhibited an initial rate of $^{22}\text{Na}^+$ uptake of about 2.3 nmol of $^{22}\text{Na}^+$ /min/culture. This value increased rapidly between days 3 and 4.5 in culture, reaching 17 nmol of $^{22}\text{Na}^+$ /min/culture, then continued to increase at a slower rate for the following few days (Fig. 2, *solid circles*). As shown in Figure 2, the slower increase in the initial rate of $^{22}\text{Na}^+$ uptake in response to both toxins

(*solid circles*) after day 4 in culture approximately parallels the increase in the initial rate of $^{22}\text{Na}^+$ uptake in response to BTX alone (*open circles*).

The $^{22}\text{Na}^+$ uptake stimulated by BTX (or veratridine) alone and that stimulated by BTX (or veratridine) plus ScTX were sensitive to tetrodotoxin (TTX) in a dose-dependent manner. As shown in Figure 3, mature (9-day-old) cultures exhibited a dose dependency for TTX which had a half-maximal effect at 4 nM TTX, a value that is in close agreement with the K_d of binding of [^3H]TTX or [^3H]STX to chick skeletal muscle (Frelin et al., 1981; Baumgold et al., 1983). The immature (day 4) cultures, however, were considerably less sensitive to TTX than the mature cultures, and exhibited a half-maximal effect at 15 nM TTX. It is unclear why the immature cultures have a K_d for [^3H]STX binding that is similar to that of mature cultures (Fig. 5, Baumgold et al., 1983), but are less TTX sensitive than mature cultures, as measured either electrophysiologically (see below) or by $^{22}\text{Na}^+$ uptake studies (Fig. 3).

Electrophysiology. Under the culture conditions used in the present study, the rapid burst of cell fusion (30 to 50 hr after plating) was followed by a period of rapid myotube growth. By 72 hr, the cultures consisted predominantly of large syncytial myotubes which had an average resting potential (RP) of about -65 mV. This large RP remained constant for the next 8 days in culture (Fig. 4B), thus making it possible to study the development of the Na^+ conductance and the effects of ScTX on this development without adjustments of the RP.

Development of the Na^+ conductance during myotube differentiation. As a result of different culture conditions, the initial and final events in the developmental time

Figure 2. Time course for the development of BTX (○)- and BTX + ScTX (●)-stimulated $^{22}\text{Na}^+$ uptake. The same data as shown in Figure 1 apply, except that the amount of $^{22}\text{Na}^+$ taken up in the absence of any drug (C, Fig. 1) has been subtracted from all values and the amount of $^{22}\text{Na}^+$ taken up by 7.5-day-old cultures in the presence of BTX + ScTX has been defined as 100%. BTX (200 nM) and ScTX (300 nM) were used. The values shown are the average of three determinations and the error bars represent the standard deviation. x represents the fusion index which was determined as described under "Materials and Methods."

Figure 3. TTX sensitivity of veratridine (200 μ M) plus ScTX (100 nM)-stimulated $^{22}\text{Na}^+$ uptake in muscle cells grown in culture. The cells were plated at an initial density of 4.2×10^4 cells/cm 2 . The uptake study was performed as described under "Materials and Methods" with the exception that the indicated concentrations of TTX were added to the toxin incubation medium. The cells were incubated in the $^{22}\text{Na}^+$ uptake medium for 30 sec. The amount of $^{22}\text{Na}^+$ taken up in the absence of drugs was subtracted from all values. Each point represents the average of three determinations whose standard deviation was usually less than 10%.

Figure 4. *A*, Changes in the maximum rate of rise ($\dot{V}_{\max}$) of the Na^+ action potential during differentiation of chick skeletal muscle in culture. $\circ$, control cultures; $\bullet$, cultures treated with 216 nM ScTX. Each point represents measurements from 20 to 25 large myotubes that displayed a maximum action potential at the site of recording. Myotubes in 5- to 8-day-old cultures that displayed passive (i.e., Fig. 5C) or small (i.e., Fig. 6A) $\dot{V}_{\max}$ at the site of recording were discarded. *B*, Changes in the resting potential as a function of time in culture.

course of the Na^+ conductance were somewhat different from those described previously (Spector and Prives, 1977). Thus, depolarizing pulses applied from the RP of long or branched myotubes in day 3 cultures elicited only

slow (maximum rate of rise <1 V/sec) and prolonged (duration = 10 to 25 sec) action potentials (APs) (Fig. 5A). These responses did not change throughout development and were usually associated with contractures of the entire myotube (Spector and Prives, 1977). They were insensitive to TTX (Fig. 7A) and could be elicited in Na^+ -free medium. No fast Na^+ component could be produced in these myotubes even if the membrane potential was hyperpolarized to -100 mV by steady inward current.

A Na^+ component of the AP that was blocked by TTX (1 μ M) and was absent in Na^+ -free medium could first be elicited in 4-day-old cultures, but the percentage of large myotubes that displayed this component was rather small (30%). Furthermore, the peak amplitude and the maximum rate of rise ($\dot{V}_{\max}$) of these Na^+ APs were poorly developed, having average values of -20 mV and 9 V/sec, respectively (Fig. 4). Both of these characteristics increased rapidly between days 4 and 6 in culture, reaching average values of about 0 mV and 150 V/sec (Fig. 4). These fast Na^+ APs could be almost completely abolished by low concentrations of TTX (30 nM) which, even at higher concentrations (2 μ M), had no effect on the prolonged AP. As previously described (Spector and Prives, 1977), during this period the myotube membrane was heterogeneous and fast Na^+ AP generation was restricted to discrete patches intercalated between regions showing only the prolonged AP (Fig. 5C). By days 7 and 8 in culture, the entire myotube acquired the ability to generate Na^+ APs. These APs exhibited large variability in peak amplitudes and $\dot{V}_{\max}$ values (Fig. 6, A and

Figure 5. Effects of ScTX (216 nM) on action potentials during muscle differentiation in culture. In all cases, recordings are from the resting potential. *A*, Voltage responses to a depolarizing current pulse of a 3-day-old myotube before (trace 1) and after (traces 2 to 4) application of ScTX. Note the time course of the changes in the slow response and the appearance of a fast action potential 9 min after addition of the toxin. *B* and *C*, Voltage responses to depolarizing and hyperpolarizing current pulses elicited from 4-day-old (*B*) and 5-day-old (*C*) myotubes. The *upper records* (labeled *CONTROL*) show slow and prolonged responses obtained before ScTX application. The *lower records* (labeled *ScTX*) were taken from the same control myotube and show the induction of a fast action potential following toxin application. Note that the peak amplitude and $\dot{V}_{\max}$ values in ScTX-treated myotubes increase as a function of time in culture. In this figure and in Figures 6 and 7, *upper traces* represent injected current (calibration: 5 nA), *middle traces* represent the voltage response (calibration: 20 mV), and *lower traces* represent the $\dot{V}_{\max}$ (calibration: 32.5 V/sec in *A*; 65 V/sec in *B* and *C*).

B), indicating that the distribution of Na^+ channels is still not homogeneous throughout the myotube membrane. Fully developed APs could be elicited from the entire myotube in 9- to 10-day-old cultures (Fig. 6C), although under our culture conditions repetitive firing and fast twitches were rarely observed either spontaneously or in response to stimulation.

Development of the Na^+ conductance in myotubes treated with scorpion toxin. The electrophysiological development of the Na^+ conductance just described shows a striking similarity to the rate of elaboration of saxitoxin-binding sites (see Fig. 8, Baumgold et al., 1983). In contrast, the elaboration of the ScTX-binding sites

proceeds at a much faster rate and differs markedly from the time course of acquisition by the myotube membrane of the ability to generate Na^+ APs. Thus, myotubes in 3- and 4-day cultures already show substantial ScTX binding but little evidence of saxitoxin binding or of a functional Na^+ conductance system. Taken together, these results strongly suggest that the number of saxitoxin-binding sites reflects functional Na^+ channels, whereas the ScTX-binding sites represent membrane components that may constitute a population of nonfunctional Na^+ channels. In order to determine what role ScTX-binding sites play in the elaboration of a functional Na^+ conductance system, we have studied the physiological consequences of this binding during myotube development.

In agreement with our binding studies (Baumgold et al., 1983), we have found that concentrations of ScTX below the dissociation constant for ScTX binding ($K_d = 62 \pm 20$ nM) did not affect the electrical activity of myotubes of any age. Small effects were seen with concentrations in the range of the K_d and these effects were enhanced in a dose-dependent manner to give optimum effects with concentrations in the range of 200 nM. This concentration range is more than 10-fold higher than that required to modify the electrical activity of neuroblastoma cells (Spector and Baumgold, 1982), indicating that this component of the Na^+ channel is different in the two preparations.

When ScTX at the optimum concentration (200 to 270 nM) was added to muscle cultures bathed in normal medium, it initiated a sequence of typical changes in electrical activity irrespective of the age of the culture. These changes did not occur in the presence of TTX (1 μM) or in Na^+ -free solutions, indicating that ScTX specifically affects Na^+ channels. As illustrated in Figure 8, this sequence usually consisted of a rapid depolarization of the membrane potential to values between -10 and 0 mV within a few seconds after toxin application, followed by a slow and sometimes irregular decline (0.5 to 2 min) to the original resting level. Following this initial response, the silent myotube became active, showing complex patterns of spontaneous responses. These responses and the responses to applied current increased in size, $\dot{V}_{\max}$, and duration over the next few minutes (Figs. 8 and 10A), and by 8 to 10 min maximum effects were reached which persisted for the duration of the experiment.

Different patterns of spontaneous activity were seen in different myotubes irrespective of their age. There were, however, some common features to all patterns of ScTX-induced spontaneous activity. In the absence of applied steady current, all ScTX-treated myotubes exhibit transitions between the resting state and a depolarized state. As illustrated in Figures 8 and 10A, the typical depolarization consisted of a fast AP followed by a prolonged plateau, lasting up to several minutes, whose configuration and duration varied from cell to cell. The plateau could be interrupted by brief and small repolarizations or by an abrupt return to the original RP. In all cases, however, the RP was more often at a depolarized state, that is, under conditions of current flow, than at the resting state. These spontaneous transitions were sensitive to changes in RP. Complete cessation of activity occurred when the cells were hyperpolarized to values more negative than -120 mV, whereas steady depolari-

Figure 6. Effects of ScTX (216 nM) on action potentials during muscle differentiation in culture. Responses obtained from 7-day (A), 8-day (B), and 10-day (C) myotubes before (*upper records*) and after (*lower records*) application of ScTX. Note in all cases the appearance of full-sized fast action potentials in the presence of ScTX, irrespective of the initial response (see the text for details). Note in B the time course of the ScTX effects.

Figure 7. Effect of TTX (100 nM) on the action potentials elicited in 9-day myotubes in the absence (A) and presence (B) of ScTX (216 nM). Note that TTX affects the fast AP alone. Note also the similarity between the TTX-insensitive response in the mature myotubes and the slow and prolonged response elicited in the immature myotube in Figure 5.

Figure 8. Sequence of effects of ScTX (270 nM) on electrical activity in a 3-day-old myotube. Control records (not shown) are similar to those in Figure 9. *Upper trace*, Immediate effects of ScTX. Note the rapid depolarization and the slow and irregular decline. *Middle trace*, Four minutes after toxin application, the myotube becomes spontaneously active, but the depolarizing phase is still slow (<1 V/sec). *Lower trace*, Spontaneous activity 9 min after toxin application. Note the sharp increase in the peak amplitude of the depolarizing phase which reaches ~ 40 V/sec. Note also the change in the pattern of the spontaneous discharge.

zation caused a voltage-dependent decrease in AP amplitude and a pronounced shortening of the prolonged plateau which resulted in a continuous and regular discharge of low amplitude spikes (Fig. 9). Such discharges could be seen even at 0 mV, or briefly, in the absence of steady depolarization at the end of the plateau response of more mature myotubes (Fig. 10A). In some cells ScTX caused a persistent depolarization that could be interrupted briefly, either spontaneously or by hyperpolarizing current pulses (Fig. 10B).

In addition to complex spontaneous discharges, ScTX induced in myotubes of all ages an increase in the peak amplitude and $\dot{V}_{\max}$ of the TTX-sensitive fast AP as well as an increase in the peak amplitude and duration of the TTX-insensitive prolonged AP. Both of these effects could be completely abolished by TTX ($1 \mu\text{M}$). However, the ScTX-modified prolonged AP, like the ScTX-induced spontaneous discharges, did not change as a function of time in culture, whereas the maximum values of the $\dot{V}_{\max}$ and of the peak amplitude of the fast AP were age dependent. As shown in Figure 4, in the presence of ScTX the $\dot{V}_{\max}$ increased sharply between days 3 and 6 in culture from an average of 35 V/sec by day 3 to an average of 220 V/sec by day 6. The time course of this increase is thus strikingly similar to that of the increase in $\dot{V}_{\max}$ in untreated myotubes, although for any given age group the average $\dot{V}_{\max}$ value in the presence of ScTX was larger by about 35 to 60 V/sec, depending on the age of the culture, than the corresponding value in the absence of ScTX. It is clear, however, that the relative

increase in $\dot{V}_{\max}$ markedly decreased as the myotube matured. Thus, for example, the $\dot{V}_{\max}$ of the full-sized AP elicited from a 10-day-old myotube (Fig. 6C) increased from about 158 V/sec to 220 V/sec after ScTX application, i.e., a 0.4-fold increase, whereas the $\dot{V}_{\max}$ of the slow response elicited from a 3-day-old myotube (Fig. 5A) increased from about 1 V/sec to 35 V/sec; i.e., a 35-fold increase.

The ScTX-induced fast AP in any given age group exhibited little variability in average peak amplitude and $\dot{V}_{\max}$ values even though control values, particularly in large 5- to 8-day-old myotubes, could vary largely from myotube to myotube due to membrane heterogeneity. Figures 5 and 6 illustrate examples of responses elicited from large branched myotubes in 5-, 7-, and 8-day-old cultures. Although myotubes at these ages have a substantial saxitoxin-binding capacity and can display full-sized APs, in the examples given, the fast AP was either absent (day 5) or poorly developed (days 7 and 8) at the site of recording. After ScTX application all of these myotubes displayed full-sized APs whose $\dot{V}_{\max}$ values were comparable to those obtained in corresponding myotubes displaying initially a full-sized AP.

The most remarkable effects of ScTX on AP generation were seen in 3- to 4-day-old myotubes which completely lacked the ability to generate a fast AP and which had little or no saxitoxin-binding capacity. Examples of the typical slow and prolonged responses elicited from these myotubes are illustrated in Figure 5. As shown, ScTX application induced the appearance of a fast AP

DAY 4

Figure 9. Effects of ScTX (216 nM) on electrical activity in 4-day myotube. The upper trace shows a control record of a silent myotube. A slow and prolonged response is elicited by a depolarizing pulse (arrows). Lower traces, Effects of membrane potential on the rate and configuration of the ScTX-induced spontaneous discharge. The adjusted membrane potential is indicated to the right of each voltage trace. Note the sharp decrease in the amplitude and the spike interval with steady depolarization.

whose peak amplitude and $\dot{V}_{\max}$ were determined by the age of the culture. The time course of the development of the fast AP in response to addition of ScTX shows that the $\dot{V}_{\max}$ and peak amplitude reached maximal values by 9 min; i.e., well after the myotube became spontaneously active (3 to 4 min). This time course is, however, independent of spontaneous activity or of the ensuing membrane depolarization since it remained unaltered when spontaneous activity was suppressed by adjusting the membrane potential to -120 mV with steady inward current. Furthermore, application of BTX (200 nM) or veratridine (200 μ M), which caused persistent depolarization, did not induce the appearance of the fast AP. Taken together, these results strongly suggest that ScTX binds to a population of membrane components that appear early in development and constitute nonfunctional sodium channels, and this binding transforms these nonfunctional channels to functional ones. Moreover, the striking parallelism in the changes in the $\dot{V}_{\max}$ during differentiation between untreated and ScTX-treated myotubes demonstrates that such a transformation can occur at all stages of development of the normal Na^+ conductance system.

Discussion

In this and in the preceding paper (Baumgold et al., 1983) we have used biochemical and electrophysiological techniques to study the expression of sodium channels during differentiation of chick skeletal muscle in culture. The data obtained show that biochemical and electrophysiological manifestations of sodium channel development are initiated shortly after cell fusion. At this stage the myotubes bind a small amount of $[^{125}\text{I}]\text{ScTX}$, take up some $^{22}\text{Na}^+$ in response to BTX + ScTX stimulation, and can generate fast action potentials only after exposure to ScTX. However, other manifestations of the sodium channel, including $[^3\text{H}]\text{STX}$ binding, BTX-stimulated $^{22}\text{Na}^+$ uptake, and fast sodium action potentials in untreated cells, are not yet evident. Subsequently, the biochemical elaboration of the sodium channel proceeds at two different rates. The $[^3\text{H}]\text{STX}$ binding and BTX-stimulated $^{22}\text{Na}^+$ uptake increase gradually to reach maximal values by around day 8. On the other hand, the $[^{125}\text{I}]\text{ScTX}$ binding and the BTX + ScTX-stimulated $^{22}\text{Na}^+$ uptake show an initial rapid increase up to around day 4.

These two rates are also reflected in the electrophysiological development and in the effects of ScTX on this

Figure 10. A, Effects of ScTX (216 nM) on the electrical activity of a 10-day-old myotube. Note the initial depolarization (*upper trace*) and the time course of the changes in the amplitude and configuration of the voltage response following application of the toxin. The *lower trace* in A shows the spontaneous activity recorded 105 min after toxin addition. Note the small spikes at the end of the prolonged plateau. B, An example of a persistent depolarization in the presence of ScTX (216 nM) recorded from a 6-day-old myotube. Note that hyperpolarizing pulses terminate the persistent depolarization.

development. In 3- or 4-day-old myotubes, a fast sodium action potential can barely be detected. However, in the presence of ScTX, these immature myotubes can generate an appreciable TTX-sensitive sodium action potential, suggesting that ScTX unmasks channels that are already present but nonfunctional. This hypothesis is supported by both the binding studies and the $^{22}\text{Na}^+$ uptake studies. Thus, immature, 4-day-old cultures exhibit substantial amounts of [^{125}I]ScTX binding and very little [^3H]STX binding. With further development in culture, the $\dot{V}_{\text{max}}$ and peak amplitude of the sodium action potential increase gradually and only at about day 9 is the entire myotube capable of generating full-sized sodium action potentials. The unmasking of nonfunctional sodium channels by ScTX proceeds, however, at a much faster rate and by day 5 in culture, the entire myotube displays full-sized sodium action potentials whose $\dot{V}_{\text{max}}$ and peak amplitude values are comparable to those obtained in 9-day-old untreated myotubes.

These findings suggest that a population of sodium channels becomes incorporated into the cell membrane in an immature, nonfunctional form capable of binding [^{125}I]ScTX alone. The fast rate of elaboration of [^{125}I]ScTX binding, BTX + ScTX-stimulated $^{22}\text{Na}^+$ uptake and the $\dot{V}_{\text{max}}$ of the ScTX-modified sodium action poten-

tial are thus a measure of the rate of incorporation of these immature sodium channels into the cell membrane. The slower rate of elaboration of [^3H]STX binding, BTX-stimulated $^{22}\text{Na}^+$ uptake, and the $\dot{V}_{\text{max}}$ of the sodium action potential of untreated cultures are measures of the rate of appearance of mature sodium channels in the cell membrane.

The relationship between the immature, nonfunctional and the mature, functional sodium channels during development is intriguing. Two alternative explanations can be advanced to account for this relationship.

1. The development of the functional sodium channel is controlled by a post-translational rather than a translational process and involves the transformation of immature nonfunctional channels to mature, functional ones.

2. There are two types of sodium channels, each controlled by a different biosynthetic pathway.

That the biochemical and electrophysiological manifestations of the immature form of sodium channel appear first and reach maximal values considerably earlier than those of the mature form suggests a conversion of one form to another by a post-translational modification or by an aggregation of pre-existing membrane components. The recent finding (Strichartz et al., 1982) that the

protein synthesis inhibitor cycloheximide is much more inhibitory to the expression of [^3H]STX binding in immature cultured muscle cells than in mature cells tends to support this hypothesis.

An alternative interpretation of our data is based on the striking parallelism in the development of the sodium action potential and of the $^{22}\text{Na}^+$ uptake between normal cultures and those treated with ScTX. This parallelism suggests that ScTX binds to and affects a different population of channels that reaches a plateau by day 4 in culture and that this population is responsible for the additional component of sodium current or $^{22}\text{Na}^+$ uptake at all stages of myotube development.

Our findings strongly suggest that the rapid (6 to 9 min) appearance of fast sodium action potentials following ScTX treatment is not due to *de novo* synthesis of the sodium channel protein, but rather to a specific modification of pre-existing membrane components by ScTX which may involve either a post-translational modification or an aggregation process. How ScTX triggers this modification and whether an analogous process occurs during development must await further studies. Recently, it has been shown that a partially purified sodium channel protein from rat brain can be phosphorylated (Costa et al., 1982). It will be interesting to determine whether this kinase-dependent phosphorylation can be activated by ScTX.

Immature sodium channels are not unique to developing excitable membranes. Nonfunctional sodium channels with similar properties have been described in a variety of nonexcitable cells (Munson et al., 1979; Romey et al., 1979; Pouyssegur et al., 1980; Frelin et al., 1982). Although the functional significance of these immature sodium channels is unclear, our results suggest that, unlike excitable cells, these cells lack a major step required for the expression of functional sodium channels.

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