

Differential Expression of Sodium Channel Activities During the Development of Chick Skeletal Muscle Cells in Culture

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ABSTRACT The expression of Na⁺ channels during differentiation of cultured embryonic chick skeletal muscle cells was investigated using saxitoxin (STX) and batrachotoxin (BTX), which previously have been shown to interact with distinct, separate receptor sites of the voltage-sensitive Na⁺ channel of excitable cells. In the present study, parallel measurements of binding of [³H]-STX (*STX) and of BTX-activated ²²Na⁺ uptake (Na influx) were made in order to establish the temporal relationship of the appearance of these two Na⁺ channel activities during myogenesis. Na influx was clearly measurable in 2-d cells; from day 3 to day 7 the maximum Na influx approximately doubled when measured with saturating BTX concentrations potentiated by *Leiurus* scorpion toxin, while the apparent affinity of BTX, measured without scorpion toxin, also increased. Saturable *STX binding did not appear consistently until day 3; from then until day 7 the *STX binding capacity increased about threefold, whereas the equilibrium dissociation constant (K_D) decreased about fourfold. Although Na influx in cells of all ages was totally inhibited by STX or tetrodotoxin (TTX) at 10 μM, lower concentrations (2–50 nM) blocked the influx in 7-d cells much more effectively than that in 3-d cells, where half the flux was resistant to STX at 20–50 nM. Similar but smaller differences characterized the block by TTX. In addition, when protein synthesis is inhibited by cycloheximide, both Na influx and *STX binding activities disappear more rapidly in 3-d than in 7-d cells, which shows that these functions are less stable metabolically in the younger cells.

INTRODUCTION

During the development and differentiation of many excitable cells, there are changes in the nature of the voltage-dependent ion permeabilities of the plasma membrane. The properties of sodium channels whose activities sub-

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serve impulse conduction are modified during the development of mammalian muscle. In neonatal rat muscle, sodium channels are quite resistant to inhibition by the drug tetrodotoxin (TTX); however, within 2–3 wk after birth, almost all of the muscle sarcolemma develops a normal sensitivity to TTX (Harris and Marshall, 1973). When rat skeletal muscle is grown in culture, the sodium channels do not develop a normal sensitivity to TTX (Kidokoro, 1975; Lawrence and Catterall, 1981*a*), but other neurotoxins do affect these channels, modifying their ion permeability properties as they do those of other TTX-sensitive sodium channels (Stallcup, 1977; Lawrence and Catterall, 1981*b*).

At least four separate sites for the binding of neurotoxins to normal sodium channels have been identified. One site binds the alkaloid activators veratridine, aconitine, and batrachotoxin (BTX), which produce a persistent sodium permeability; a second site binds one class of polypeptide toxins from scorpions or sea anemones, and prevents sodium channel inactivation and potentiates the action of the activators (Catterall, 1980*a*); a third site binds another type of scorpion toxin that modifies channel closing by the reversal of the activation step (Cahalan, 1975; Wang and Strichartz, 1983); and a fourth site binds the nonprotein toxins TTX and saxitoxin (STX), which block the activator-induced sodium permeability in a noncompetitive manner (Catterall, 1975; Stallcup, 1977), apparently by binding near the outer opening of the channel.

In this paper we report results of a study on the time course of appearance in chick skeletal muscle cells in culture of two sodium channel-related activities, STX binding and BTX-activated Na⁺ influx. Unlike rat muscle cells, these chick cells do develop a TTX-sensitive sodium channel (Kano et al., 1972; Spector and Prives, 1977; Catterall, 1980*b*; Frelin et al., 1981). Furthermore, in this system the synchronous nature of myogenesis *in vitro* permits a coherent measure of the rate and extent of expression of the distinctive Na⁺ channel components. Our findings show that the appearance of STX binding sites follows a different time course from that of BTX-activated channels. The maturation of both components is characterized by changes in the affinities of these components for their respective ligands and by an increase in their metabolic stability, as indicated by experiments using cycloheximide, an inhibitor of protein synthesis. A brief report of some of these findings has been presented previously (Strichartz et al., 1982).

MATERIALS AND METHODS

Muscle Culture

Primary cultures of embryonic skeletal muscle were prepared from the breast of a 12-d-old chick embryo, as previously described (O'Neill and Stockdale, 1972; Paterson and Prives, 1973). Cells were plated on collagen-coated 6-cm culture dishes at an initial density of 1.8×10^6 cells per dish and grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 25 mM Hepes (pH 7.4), 10% (vol/vol) horse serum, and 2% (vol/vol) chicken embryo extract under an atmosphere of 90% air-10% CO₂. 48 h after plating, cultures were fed with growth medium containing

cytosine arabinoside (10 μ M) for a 48-h period to minimize fibroblast proliferation (cf. Fischbach and Cohen, 1973).

Measurement of $^{22}\text{Na}^+$ Uptake

Rates of $^{22}\text{Na}^+$ uptake were measured essentially as described previously by Catterall (1977, 1980b). Cultures were preincubated with the concentrations of neurotoxins indicated in the text or figure legends in 1 ml of Na^+ -free medium consisting of 135.4 mM KCl, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM MgSO_4 , 0.1 mM ouabain, and 1 mg/ml bovine serum albumin (BSA) for 30 min at 37°C. At the end of this period, medium was removed and cells were rinsed twice within 10 s with 2.5 ml of a medium consisting of 5.4 mM KCl, 130 mM choline chloride, 50 mM Hepes (pH 7.4), 5.5 mM glucose, 0.8 mM MgSO_4 , and 1 mg/ml BSA at 37°C. Uptake of $^{22}\text{Na}^+$ was then assayed at 37°C in 1 ml of medium containing the same concentrations of neurotoxins, 5.4 mM KCl, 120 mM choline chloride, 10 mM NaCl, 50 mM Hepes (pH 7.4), 5.5 mM glucose, 0.8 mM MgSO_4 , 0.1 mM ouabain, and $^{22}\text{NaCl}$ (2 $\mu\text{Ci/ml}$). Under these conditions, $^{22}\text{Na}^+$ uptake activated by BTX is linear with time for 1 min (Fig. 1). In most of the reported experiments, $^{22}\text{Na}^+$ uptake was assayed after 30 s, being terminated by washing five times within 25 s with 2 ml of ice-cold medium consisting of 163 mM choline chloride, 50 mM Hepes (pH 7.4), 5.5 mM glucose, 0.8 mM MgSO_4 , 1.8 mM CaCl_2 , and 1 μM TTX. This procedure was followed to establish and control as similar a "resting potential" as possible in cells of all ages and with many different drug conditions. By minimizing the external sodium concentration and permitting equilibration of the cytoplasm with high K^+ during the preincubation period in the presence of an inhibitor of the (Na^+ - K^+) pump, we established the membrane potentials during the flux measurements as close as possible to a Nernstian value of E_K equal to -88 mV at the $[\text{K}^+]$ we used. We made no effort to measure these membrane potentials, but at -88 mV they would be more negative than those reported for the same type of cells in normal incubation medium, -41 to -60 mV (Spector and Prives, 1977) and -60 to -70 mV (Frelin et al., 1981).

In experiments using scorpion toxin, cultures were preincubated in 1 ml of Na^+ -free medium consisting of 5.4 mM KCl, 130 mM choline chloride, 50 mM Hepes (pH 7.4), 5.5 mM glucose, 0.8 mM MgSO_4 , 1 mg/ml BSA plus 300 nM scorpion toxin plus BTX, as noted, for 30 min at 37°C. Cells were not preincubated in high- K^+ solutions with scorpion toxin present because these toxins bind with an affinity that is reduced in depolarized membranes (Catterall, 1980b). Saturating scorpion toxin concentrations were used, however, so that any small differences in membrane potential would not compromise the efficacy of this drug. $^{22}\text{Na}^+$ uptake was assayed at 37°C in 1 ml of a medium consisting of 5.4 mM KCl, 10 mM NaCl, 120 mM choline chloride, 50 mM Hepes (pH 7.4), 5.5 mM glucose, 0.8 mM MgSO_4 , 1 mM ouabain, $^{22}\text{NaCl}$ (2 $\mu\text{Ci/ml}$), and supplemented with neurotoxins as noted. Cells were suspended in 1 N NaOH containing 1% Triton X-100, and radioactivity was determined by gamma spectroscopy.

In all experiments, both with and without scorpion toxin, $^{22}\text{Na}^+$ uptake in the absence of all neurotoxins was subtracted from the total uptake to yield the net specific uptake occurring through activated sodium channels. Results are expressed as nanomoles of $^{22}\text{Na}^+$ taken up per minute per culture plate.

Measurement of Saxitoxin Binding

Tritiated saxitoxin (*STX) was prepared and purified by the method of Ritchie et al. (1976). *STX uptake was assayed in suspensions of cell fragments. The plated

cultures were first washed free of growth medium with Locke solution (154 mM NaCl, 2 mM KCl, 2.2 mM CaCl₂, and 5 mM Hepes, pH 7.2). Cells were then scraped from the plates with a Tygon-tipped rod and uniformly suspended in ~3.0 ml of Locke solution using a glass-Teflon homogenizer stroked 7–10 times by hand. All manipulations on cells beginning with the removal of growth medium were conducted at 0–4°C.

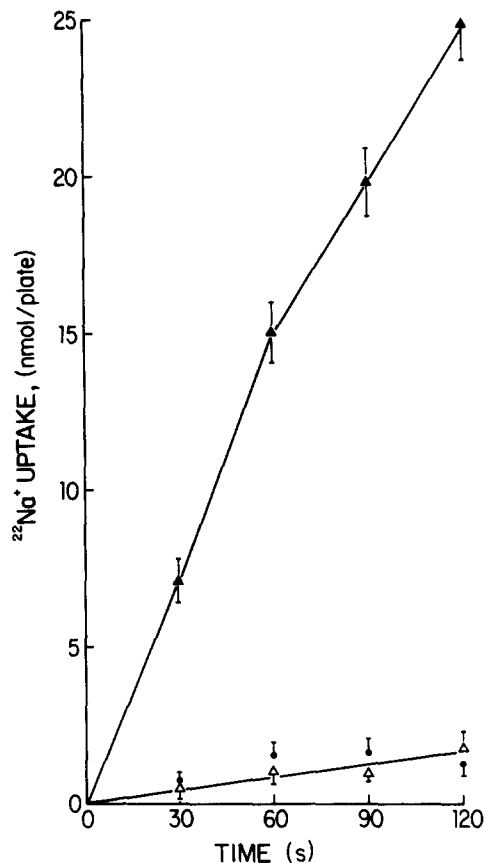


FIGURE 1. Kinetics of ²²Na uptake by muscle cells at 5 d post-plating. Cells were pre-incubated in Na⁺-free medium for 30 min at 37°C without (●) or with (▲) 1 μM BTX or with 1 μM BTX plus 1 μM TTX (Δ). Intracellular ²²Na content was assayed at the times indicated, as described in Materials and Methods. Data points are placed on the average values of three determinations and the vertical bars span the range of these values.

*STX, at the concentrations indicated in the text, was added to cell homogenates for an incubation period of 90 min at 4°C. Incubations of 60 min gave very similar results. The suspended membranes (1.0 ml) were then centrifuged at 12,000 *g* for 3 min (Microfuge B; Beckman Instruments, Inc., Fullerton, CA), forming a tightly packed pellet at the bottom of a small polystyrene centrifuge tube. The supernatant was decanted and saved for counting to determine the free *STX concentration, and

the inside of the tube containing the pelleted membranes was rapidly washed out with ice-cold Locke solution. All liquid remaining in the tube was wicked away, leaving the pellet free of any superficial toxin-containing solution. Tissue solubilizer (0.2 ml Protosol; New England Nuclear, Boston, MA) was added to the pellets for digestion during an overnight period at room temperature in capped tubes. Radioactivity was measured by liquid scintillation spectroscopy in a Beckman model 8100 LS spectrometer using 5.0 ml Dimiscint (National Diagnostics, Inc., Somerville, NJ) scintillation fluid. All STX uptakes are expressed as femtomoles per plate with values of the mean \pm SEM; triplicate measurements of *STX uptakes were always performed.

The typical uptake of increasing concentrations of *STX by a suspension of 7-d muscle cells is graphed in Fig. 2A. As in many other excitable membranes, the total *STX uptake at equilibrium is the sum of a component that increases linearly with *STX concentrations (U_{lin}) and a component that saturates following a hyperbolic (Langmuir) binding curve (U_{sat}):

$$U_{total} = U_{lin} + U_{sat} \quad (1)$$

$$= b[*STX] + U_{max} \frac{[*STX]}{K_D + [*STX]},$$

where b is a constant characteristic of the tissue and the method of binding assay, U_{max} is the total number of STX receptors, and K_D is the equilibrium dissociation constant for saturable STX binding. In the presence of high concentrations of a competitive inhibitor (e.g., TTX), saturable binding is essentially abolished and only the linear uptake remains: the coefficient of linear uptake, b , is determined by fitting the individual data points by a straight line using a least-squares linear regression analysis.

The number of high-affinity STX receptors can be determined by analyzing the data for U_{max} , as in the Scatchard plot shown in Fig. 2B. A straight line is fit to the data of the Scatchard plot by least-squares linear regression analysis. As for the linear uptake fit mentioned above, such analyses were performed using statistics programs contained in a programmable pocket calculator (TI-59; Texas Instruments Inc., Houston, TX). In this particular experiment, using 7-d cells, the U_{max} value is 16.1 fmol/plate, shown by the x -axis intercept. The K_D value, equal to $-\text{slope}^{-1}$ of the Scatchard graph, is 1.16 nM.

Removing the cells from the culture plates appears to have had no measurable effects on U_{max} . In one experiment we incubated 7-d cells still attached to the culture plates, on ice, with *STX in Locke solution, then washed the plates rapidly (within <10 s) with ice-cold Locke solution containing no *STX, and immediately treated the cells on the plates with 0.5 ml Triton X-100 (1%) in 1 N NaOH. The lysed cell suspension was transferred *in toto* to scintillation vials, 10 ml of scintillant was added, and the contained ^3H was measured. Average U_{max} values were equal to those measured by the pellet method described above, although the variance was far greater ($\pm 30\%$ of the mean). U_{lin} values were about twice those measured in pellets. For these reasons, we chose to measure uptake exclusively in pelleted suspensions of cell fragments.

Because of the relatively large size of the linear component of uptake, small saturable binding activities are difficult to estimate quantitatively. For this reason, with the range of U_{max} values in these cells, receptors with K_D values for STX greater than ~ 20 nM are essentially impossible to detect. Furthermore, data obtained in

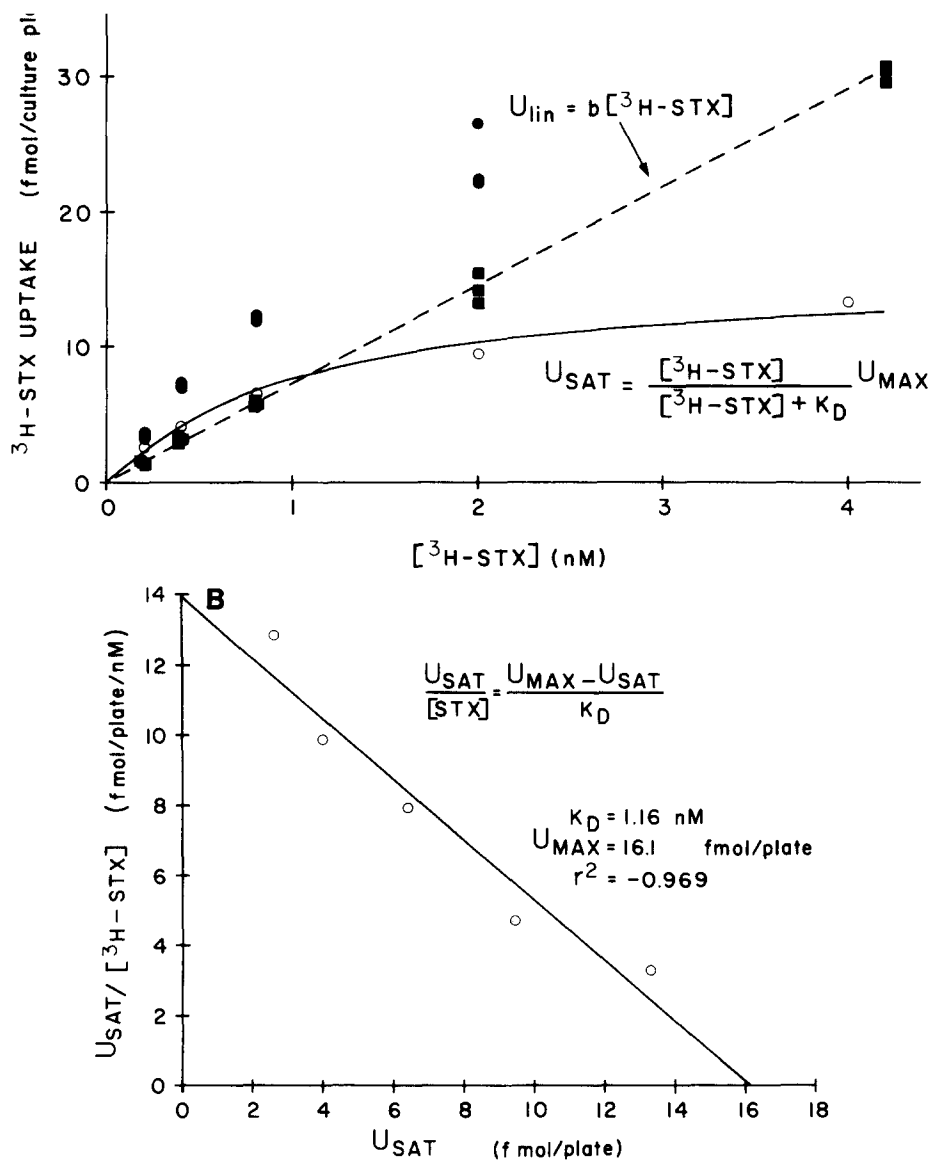


FIGURE 2. [^3H]STX uptake by 7-d cultured muscle cells. (A) Each of the triplicate measurements of [^3H]STX ($^3\text{H-STX}$) uptake is shown for the saxitoxin concentration noted on the abscissa. (●) Total uptake of $^3\text{H-STX}$; (■) linear uptake of $^3\text{H-STX}$ (U_{lin}), measured in the presence of 10^{-6} M unlabeled TTX; and (○) U_{sat} , the specific, saturable binding of $^3\text{H-STX}$, equal to the difference between the average values of total uptake and linear uptake. The curve for saturable binding is drawn from Eq. 1 (see Materials and Methods) using the parameters determined from the Scatchard plot in B. (B) Scatchard plot of the saturable $^3\text{H-STX}$ binding data from A. The line is drawn from a least-squares linear regression analysis. $T = 4^\circ\text{C}$. Incubation time = 95 min.

young cells always showed a lower relative U_{sat} than in older cells (see Table I). To maximize the average ratio of saturable binding to linear uptake during the course of development and thereby optimize the detection of high-affinity receptors, we followed the uptake of *STX at 4 nM, measuring total and linear uptake at this concentration and calculating the difference.

Protein Content

The total protein content of the cells was determined by the method of Lowry et al. (1951). Protein was measured in cells in culture from day 1 to day 9, for three separate platings, and ranged from $189 \pm 35 \mu\text{g}/\text{plate}$ on day 1 to $631 \pm 109 \mu\text{g}/\text{plate}$ on day 9. Of most pertinence to this study, the average values were $537 \pm 71 \mu\text{g}/\text{plate}$ in 3-d cells and $643 \pm 87 \mu\text{g}/\text{plate}$ in 7-d cells, corresponding to a net difference of 19.7% protein between these developmental stages.

TABLE I
Development of Saxitoxin Binding Activity

Culture age	4 nM [³ H]STX		
	U_{total}	U_{lin}	U_{sat}^*
<i>d</i>	<i>fmol/plate</i>		
2	7.16±0.04	6.21±0.04	0.95 [‡]
3	28.9±0.28	25.3±0.55	3.6 [‡]
4	31.7±1.3	26.8±0.030	4.9 [‡]
5	35.4±0.74	27.1±1.4	8.3 [‡]
6	43.3±1.2	31.6±0.70	11.7 [‡]
7	40.8±1.28	25.4±0.44	15.4 [‡]

* $U_{\text{sat}} = U_{\text{total}} - U_{\text{lin}}$. Each value of U_{total} and U_{lin} is the average of three separate measurements \pm SEM from a single plating of cells.

Significance of difference between U_{total} and U_{lin} .

[‡] $P < 0.001$.

[‡] $P < 0.005$; P values calculated from Student's two-tailed t test.

Materials

TTX was purchased from Calbiochem-Behring Corp., La Jolla, CA; ¹²⁵I-labeled α -bungarotoxin and ²²NaCl were obtained from New England Nuclear. Batrachotoxin was kindly provided by Dr. John Daly, National Institutes of Health, Bethesda, MD. *Leiurus quinquestriatus* scorpion venom was generously donated by Dr. Ging Kuo Wang, Harvard Medical School, Boston, MA. Other chemicals and drugs were purchased from Sigma Chemical Co., St. Louis, MO. Muscles cultures were prepared from Single Comb white leghorn eggs, from Westbrook Farms, Bohemia, NY.

RESULTS

The differentiation of muscle cells displays a high degree of synchrony under the culture conditions used in this study. Fusion of myoblasts into myotubes increases from $\sim 15\%$ to a maximum of 85% during the period from 24 to 48 h after plating and is accompanied by a sharp increase in the acetylcholine receptor level, as measured by the specific binding of ¹²⁵I- α -bungarotoxin (Fig. 3) (Prives and Paterson, 1974). No increase in cell population occurs after this period, because of the presence of 10 μM cytosine arabinoside.

The existence of voltage- and drug-sensitive sodium channels has been demonstrated in these cells at times as early as 2–3 d after plating (Spector and Prives, 1977; Catterall, 1980*b*; Frelin et al., 1981). We have elaborated on these results by examining the changes in pharmacological properties of sodium channels during the first 7 d after plating. Channels were characterized by their Na^+ influx, as catalyzed by the activator batrachotoxin (BTX), and by both the sensitivity of this flux to low concentrations of the channel-blocking toxins STX and TTX and the binding activity of radiolabeled STX. The constant value of the rate of BTX-catalyzed $^{22}\text{Na}^+$ influx measured over the first 30 s of exposure of $^{22}\text{Na}^+$ (see Fig. 1), under conditions of a

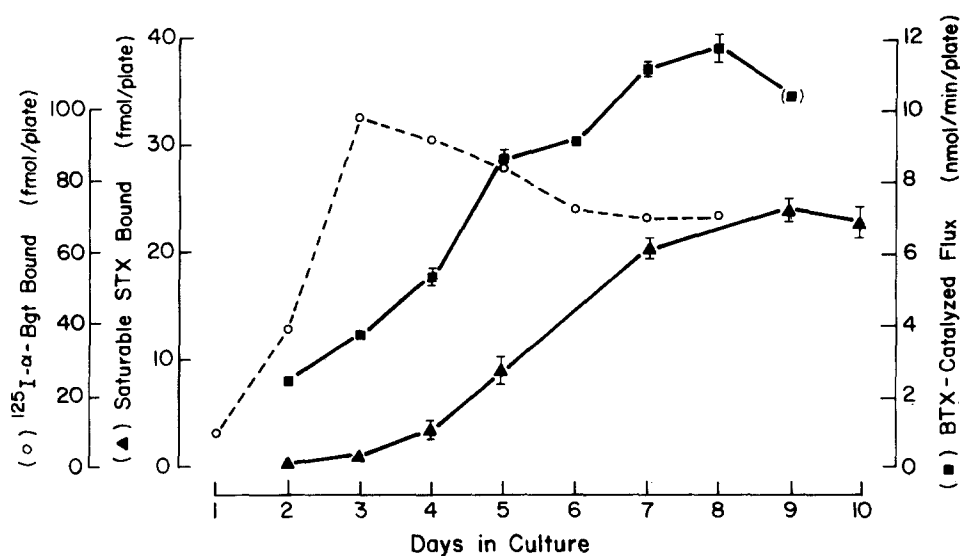


FIGURE 3. Time courses for the appearance of sodium channel-related activities in cultured muscle cells. (▲) Saturable *STX binding, measured at 4 nM *STX ; (■) Na influx catalyzed by 1 μM BTX (minus influx in 0 BTX). The points show average values of two to four determinations each made on two to four separate cell platings; vertical bars indicate SEM, which did not exceed the dimensions of the symbol where no bar is drawn. (Flux at 9 d, shown in parentheses, is from a single plating.) The binding of radiolabeled α -bungarotoxin to a different plating of cultured muscle cells is also shown.

membrane potential constant in time and among different age cultures (see Materials and Methods) and expressed as nanomoles Na^+ per minute per plate, is proportional to the steady state sodium permeability produced by activator drugs and inhibited by TTX and STX (Catterall, 1975, 1980*a*; Henderson and Strichartz, 1974). Thus the Na influx is a product of the channel number per plate, the permeability per channel, and the extent of pharmacological activation of sodium channels during development.

STX binding was also measured in these cultured muscle cells. A saturable component of STX binding can be resolved from the total uptake (see Materials and Methods) and fully characterized by specification of a binding capacity (U_{max}) and an equilibrium dissociation constant (K_D). Such binding

can be detected for sodium channels that have a high affinity for saxitoxin (Henderson et al., 1973; Ritchie et al., 1976). A nonsaturable or linear uptake of STX also occurs in all tissues; this linear uptake (U_{lin}) results from STX distributed into interstitial spaces and within the diffuse double layer adjacent to all negatively charged membranes (Ritchie and Rogart, 1977; Strichartz, 1982). In the binding assay used here, the magnitude of U_{lin} often exceeded that of U_{max} for 7-d cells when the free [$*STX$] was $>1-2$ nM (see Fig. 2A). This imbalance was even more disproportionate in younger (3-d) cells (see Tables I and III). Therefore, we chose to follow initially the uptake of STX during development at one [$*STX$] (4 nM) that was less than saturating to minimize errors caused by the very large contribution of the linear uptake.

Sodium influx, catalyzed by 10^{-6} M BTX, and saturable $*STX$ binding, measured at 4 nM $*STX$, were followed in muscle cells in culture from day 2 to day 9. Fig. 3 shows data summarized from two to four separate cells platings. Both $*STX$ binding and Na influx increase during development, both changes arising from increases in toxin affinities and receptor numbers (see below). In 2-d cells, Na influx was clearly detectable; the flux increases by ~ 4.5 -fold by day 7. In contrast, $*STX$ binding at 4 nM was not consistently detectable in 2-d cells, but was measurable in 3-d cells and had reached a plateau value between days 7 and 9 (Fig. 3). That the vanishingly small binding of $*STX$ in 2-d cells is not a consequence of limitations in the sensitivity of the binding assay, despite the relatively large linear uptake, is documented by the data in Table I, which show the results of $*STX$ uptake by a single plating of cells followed from days 2–7. A small but significant ($0.001 < P < 0.005$) difference between U_{total} and U_{lin} is discernable at day 2, demonstrating miniscule but measurable saturable binding. However, the cells at 2 d in culture from another plating showed no significant saturable binding ($U_{total} = 14.5 \pm 0.43$ fmol/plate [3]; $U_{lin} = 14.5 \pm 0.17$ fmol/plate [3]), although at 3 d a saturable binding almost equal to that listed for the 3-d cells in Table I was observed. It appears that the saturable binding of $*STX$ at 4 nM is at a vanishingly small value in 2-d cells, but grows to progressively higher levels at day 3 and beyond. By comparison, linear $*STX$ binding has reached almost a constant value by day 3.

Several different factors could account for the developmental changes in sodium channel activities graphed in Fig. 3. These include changes in the permeability of a single drug-activated channel, in the number of channels on the membrane surface, in the fraction of channels that bind STX with high affinity (K_D of <20 nM instead of >500 nM), and in smaller changes of the high affinities of the BTX and STX receptors for their respective toxins. We have resolved some of these factors by investigating (a) maximum Na influxes, (b) dose-response curves for [BTX] vs. Na influx, (c) inhibition of Na^+ influx by STX and TTX, and by characterizing saturable $*STX$ binding, all in younger (3-d) and older (7-d) cells in culture.

The relationship between [BTX] and Na influx differs between 3-d and 7-d cells. Fig. 4 illustrates this dose-response relationship for three separate cell platings. The flux values at 10^{-6} M BTX, when expressed on the basis of cell protein, are 9–11 nmol/min·mg protein for 3-d cells and 19–23 nmol/min·

mg protein for 7-d cells, values that are, respectively, about equal to those reported for 4-d cells by Frelin et al. (1981), 8 nmol/min·mg protein, and lower than that measured by Catterall (1980*b*) in 6-d cells, 56 nmol/min·mg. As in these previous studies, concentrations of BTX up to 10^{-5} M do not saturate the activated Na influx. Nevertheless, comparison of the relative Na influx in 7-d and 3-d cells over the range of [BTX] used shows that the potency of this activator increases during development. This conclusion follows from the observed decrease in the ratio of flux in 7-d cells to that in 3-d cells as a function of [BTX] (Fig. 4). The ratio falls from >5 at 10^{-7} M

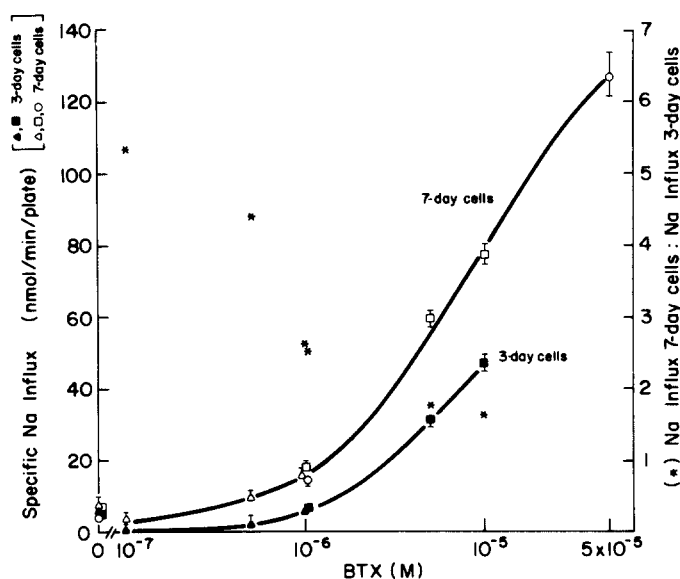


FIGURE 4. The dependence of specific ^{22}Na influx rate on BTX concentration. Triplicate measurements were made on three different platings of cultured cells at 3 d (\blacktriangle , \blacksquare) and 7 d (\triangle , \square , \circ). Initial flux rates were measured as described in Materials and Methods. The vertical bars are the sums of the standard errors of the mean from the total Na influx plus the nonspecific Na influx. The symbols placed on the abscissa at [BTX] = 0 show the magnitude of the nonspecific flux. Curves are fitted by eye. The ratio of the uptake rate of 7-d cells to that of 3-d cells is also plotted (*).

[BTX] to between 1.5 and 2 as [BTX] approaches its saturating value. Were the channel affinity for BTX the same in 3-d and 7-d cells, and only the number of channels in the membrane or the permeability per activated channel increased, then the flux ratio would not vary with BTX concentration. However, the decreasing flux ratio with increasing [BTX] reveals that the relative activation of channels in older vs. younger cells is greatest at low [BTX] and thus that, on average, channels in 7-d cells have a greater affinity for BTX than those in 3-d cells.

The statistical significance of these flux ratios is dependent on the relative size of the specific Na influx and on the size of the standard errors. At 10^{-7}

M BTX the "nonspecific" Na influx, measured with no BTX, is a large fraction of the total flux, leaving the specific Na influx as a smaller remainder. However, the spread of the measured flux values in a single plating is small, as reflected by the standard error of the mean of triplicate measurements; for example, for 10^{-7} M BTX, in 7-d cells total Na influx equaled 10.8 ± 0.49 , whereas nonspecific Na influx equaled 7.38 ± 0.97 nmol/min·plate; in 3-d cells of the same plating, the same BTX concentration activated a total Na influx of 6.20 ± 0.07 and a nonspecific influx of 5.56 ± 0.16 nmol/min·plate. The sum of these standard errors is shown as the vertical bar on each specific Na influx value in Fig. 4. As seen in this figure, for increasing [BTX], the relative error falls as the relative specific flux increases.

The [BTX] flux curves drawn in Fig. 4 for the two cell ages cannot be superimposed by simple shifts along the concentration axis alone, and detailed differences in the shapes suggest that a population of channels with higher affinity for BTX ($K_D \sim 5 \times 10^{-7}$ M) is detectable only in 7-d cells and, in addition, that the total maximum flux (F_{max}) is greater in 7-d than in 3-d cells.

This latter suggestion is supported by the results of flux experiments conducted in the presence of a scorpion toxin, which permitted the measurement of F_{max} in cells of both ages. A purified toxin from the scorpion *Leiurus quinquestriatus* (LQ II α) prevents normal inactivation of neuronal sodium channels (Wang and Strichartz, 1983) and potentiates the effects of activator drugs (Catterall, 1977, 1980a). In the presence of this toxin, BTX at 5×10^{-7} and at 10^{-6} M catalyzed a saturating Na influx of 50 and 95 nmol/min·plate in 3-d and 7-d cells, respectively (Table II), which shows that older cells have about twice the maximum pharmacologically activated sodium permeability of younger cells. This flux ratio is comparable to the flux ratio value approached at increasing concentrations of BTX in the absence of *Leiurus* toxin (Fig. 4). Furthermore, the values in Table II of specific flux at 5×10^{-7} M BTX compared with that at 10^{-6} M BTX are lower in 3-d cells (0.61) than in 7-d cells (0.72), representing yet another observation of difference of BTX potency with age in culture. Thus, during development in culture there is an increase in the apparent affinity of BTX for its receptor, accompanied by a rise in either the total number of drug-activatable channels, the Na⁺ permeability per activated channel, or both.

Binding parameters for *STX were also characterized in 3-d and 7-d cells. Table III summarizes these data from three separate platings for each culture age. Over this developmental period, the number of high-affinity STX receptors increases threefold, while the measured affinity for the toxin (K_D^{-1}) also increases, by about fourfold. When expressed in terms of total cell protein, the STX binding capacity is 17.2 ± 7.8 fmol/mg protein and 44.7 ± 7.3 fmol/mg protein in 3-d and 7-d cells, respectively. These values are comparable to those reported for TTX receptors by Frelin et al. (1981) for cells 3.3 d in culture (22 fmol/mg protein) and 4.2 d in culture (25 ± 5 fmol/mg protein). (As noted above, linear *STX binding does not change between days 3 and 7.)

TABLE II
Facilitation of BTX-catalyzed Na⁺ Influx by a Scorpion Toxin

Culture age	[BTX]	Without <i>Leiurus</i> scorpion toxin			With <i>Leiurus</i> scorpion toxin*	
		0	5.10 ⁻⁷	10 ⁻⁶	5.10 ⁻⁷	10 ⁻⁶
<i>d</i>	<i>M</i>					
3	Total Na influx	7.0±0.4	10.6±0.6	12.9±0.4	57.0±0.8	58.5±0.7
	Specific Na influx [‡] (nmol/min·plate)	—	3.6	5.9	50.0	51.5
7	Total Na influx	5.4±1.2	16.8±0.3	21.0±1.2	99.5±5.2	101.6±3.6
	Specific Na influx [‡] (nmol/min·plate)	—	11.3	15.6	94.1	96.2

Each value is the average of three separate uptake measurements ± SEM.

* *Leiurus* toxin IIα (200 nM) alone had no catalyzing activity on Na influx.

‡ Total Na influx minus Na influx in zero BTX.

The rise in the number and in the toxin affinity of STX receptors is paralleled by an increase in the potency with which STX inhibits Na influx (Fig. 5A). High concentrations (1–10 μM) of TTX and STX block all specific Na influx. The Na⁺ flux into 7-d cells is uniformly inhibited by STX, as if all the channels bound STX with a K_D of 1.2–1.6 nM. (The dissociation constant is calculated from the equation for saturable binding: β = relative STX receptor occupancy = $\frac{[STX]}{[STX] + K_D}$. Thus, $K_D = \frac{[STX](1 - \beta)}{\beta}$, where β is equal to the percent inhibition of control Na influx.) In 3-d cells about half of the Na influx persists at an STX concentration of 50 nM, the remaining flux being inhibited by the toxin as if channels bound STX with a K_D of 7–16 nM. Comparing the relative blocks of 3- and 7-d cells at 2 and 20 nM STX, and accounting for the accumulated standard errors in the determination of percent inhibition (see legend to Fig. 5), reveals that the difference in susceptibility to STX with cell age is significant at 2 nM STX ($0.005 < P < 0.01$) and at 20 nM STX ($0.005 > P$) by Student's two-tailed *t* test. A similar, although much less dramatic, difference with cell age is seen in the inhibition by tetrodotoxin (Fig. 5B). As with STX, flux into 3-d cells is more resistant to inhibition by TTX than is the flux into 7-d cells. It appears that in these

TABLE III
*STX-saturable Binding Parameters

Culture age	K_D	U_{max}
<i>d</i>	<i>nM</i>	<i>fmol/plate</i>
3	5.06±2.3	9.3±4.2
7	1.17±0.18	28.6±4.7

Values are averages ± SEM from three separate Scatchard analyses of saturation binding curves on each culture age from three different platings (see Fig. 2).

The significance of the parameter differences, evaluated by application of Student's two-tailed *t* test, is $0.05 < P < 0.10$ for the K_D values, and $0.01 < P < 0.025$ for the U_{max} values.

cells the properties of the Na^+ channel that account for the binding of STX are not identical to those that determine TTX binding, an observation that is in agreement with results from other electrophysiological studies (see Discussion). Despite the marked developmental changes in the sensitivity of Na^+ influx to low concentrations of STX, we emphasize that all of the BTX-catalyzed Na^+ influx in cells of all ages is inhibited by high concentrations of STX or TTX (10^{-6} M). All of the channels, regardless of cell age, possess receptors for these blocking toxins, but the potency of these toxins increases during differentiation.

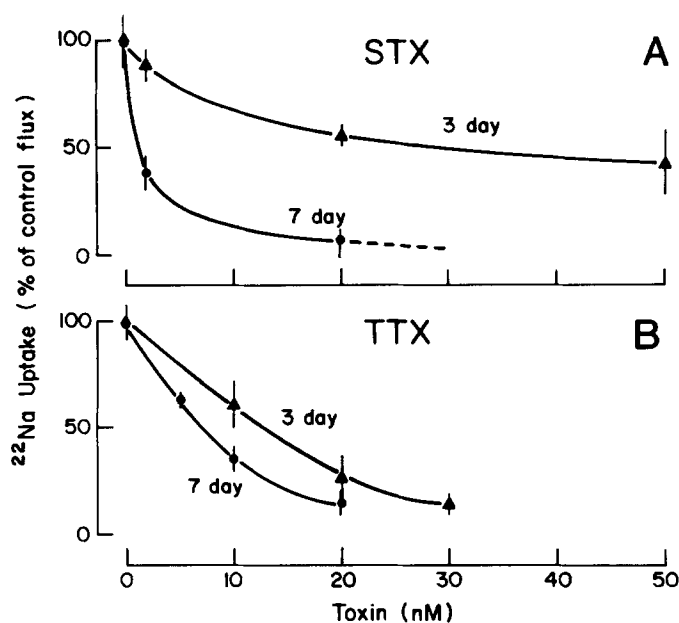


FIGURE 5. Inhibition of Na influx by increasing concentrations of STX and TTX. (A) ^{22}Na influx, catalyzed by $1 \mu\text{M}$ BTX, in 3-d (▲) and 7-d (●) cells is inhibited by STX at concentrations of 2–50 nM. (B) In identical cultures the Na influx is inhibited by TTX at 5–30 nM. Data points show the average values of two to three measurements. The vertical error bars indicate the sum of the standard errors of the mean of the directly measured total uptake (SEM = 4–7%) plus the nonspecific flux (SEM = 3%) plus the control specific uptake with no STX or TTX, which includes the two previously mentioned sources of error; (SEM = 8–12%).

To compare the metabolic stability of Na^+ channels expressed at different stages of development, 3-d, 4-d, or 7-d cells were treated with the protein synthesis inhibitor cycloheximide (CHX; $10 \mu\text{g}/\text{ml}$). Under the culture conditions we used, this concentration of CHX blocks the incorporation of labeled amino acids into protein by 95% in the first 10 min after addition of the inhibitor (unpublished observation). Since the accumulation of Na^+ channels represents the balance between channel synthesis and channel degradation, the decrease in STX binding and Na influx observed under

conditions of protein synthesis blockade (Table IV; Fig. 6) reflects the metabolic stability of Na⁺ channels. As shown in Table IV and Fig. 6, the fractional decrease of both Na⁺ uptake and STX binding, measured 24 h after interruption of protein synthesis, is greater in 3-d and 4-d cells than in 7-d cells. These results suggest that the metabolic stability of Na⁺ channels expressed on the muscle surface increases during differentiation. Because there may be a population of post-translational precursors of channels or their structural subunits, which continue to appear on the muscle cell surface even after the cessation of protein synthesis, the measured loss of channel-related activities represents a minimal rate for the true kinetics of disappearance of these functions (see Discussion).

TABLE IV
Effects of Cycloheximide on Na⁺ Channel Activities During Muscle Development in Culture

Culture age	Percent of activities in cells before the addition of cycloheximide	
	BTX-catalyzed ²² Na uptake	[³ H]STX binding
<i>d</i>		
3, 4	59±9 (3)	32±10 (2)
7	78±3 (2)	91±3 (2)

Cultures, at the ages specified, were incubated for a 24-h period in growth medium containing cycloheximide (10 µg/ml). BTX- (10⁻⁶ M) catalyzed ²²Na uptake and saturable binding of [³H]STX (4 nM) were then measured in treated cultures as described (see Materials and Methods). Results are expressed as percent of activities in untreated cells of the same plating before CHX addition and represent the average from the number of separate experiments, noted in parentheses, ± the range.

If the cells were allowed to recover for 24 h after the removal of CHX, the channel-related activities again increased (Fig. 6), which shows that the interruption in the developmental program was temporary and reversible. *STX binding recovered more rapidly than Na influx after CHX removal from 3–4-d cells, a result that parallels the different rates of increase of these two activities during this period of normal development in culture (Fig. 3).

The fractional decrease in channel activities was much smaller in 7-d cells treated with CHX. *STX binding fell by only ~9% and Na influx by ~22% compared with control values measured both in cells before CHX exposure (Fig. 6) and in untreated cells at day 8. Thus, the overall *STX binding activity appears to be more labile than the Na influx in 3-d cells but more stable in 7-d cells. From our experiments, conducted at single toxin concentrations, we cannot discriminate losses in receptor number from decreases in toxin affinity as factors contributing to the observed fall in *STX binding and in Na influx. Further studies are required to assess the contributions of changes in these parameters.

DISCUSSION

Differentiation of muscle cells in culture is accompanied by the expression of cell surface components that mediate membrane excitability (De Haan, 1980; Nelson, 1975). These components include acetylcholine receptors involved in neuromuscular transmission, and voltage-sensitive Na^+ channels responsible for the initiation and propagation of action potentials. STX and BTX have been shown to interact with distinct and separate sites on the Na^+ channels. In the present study we have established the temporal relationship

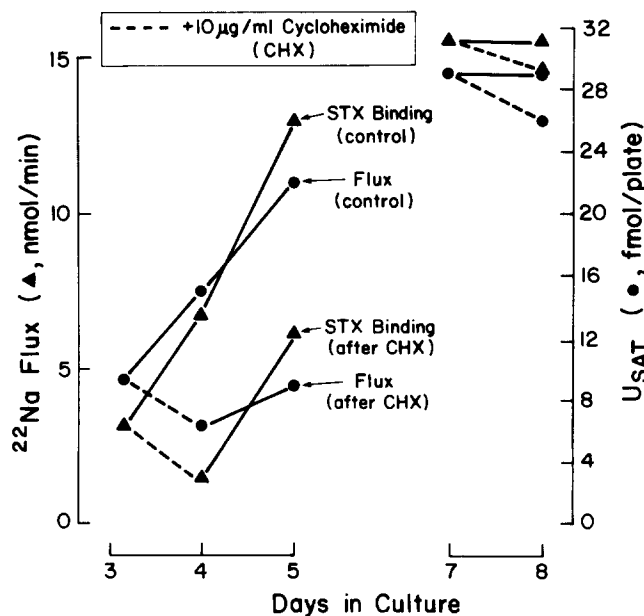


FIGURE 6. Relative stability of sodium channel-related activities in cells with inhibited protein synthesis. Measurements of saturable *STX binding (●, at 4 nM *STX) or Na influx (▲, at 1 μM BTX) in cells not growing in cycloheximide are spanned by the solid lines. The same measurements in cells growing in cycloheximide (10 $\mu\text{g}/\text{ml}$) are spanned by the broken line. Cycloheximide was washed away before all assays were conducted. Triplicate measurements of each activity were made on a single plating for 3–5-d cells and on a different plating for 7–8-d cells.

of the appearance of two Na^+ channel activities using measurements of *STX-specific binding and BTX-activated Na^+ uptake. Our results illustrate that the developmental expression of Na^+ channels is accompanied by changes in pharmacological and metabolic properties of channel components.

The synchronous nature of differentiation obtained under the culture conditions used here has allowed us to determine the time course of appearance of Na^+ channel activities (Fig. 3). In the early stages of development, days 2–3, BTX-induced Na^+ influx precedes the appearance of specific STX

binding. During subsequent differentiation both Na^+ influx and specific binding of [^3H]STX increase markedly. This increase is comprised of increments in number as well as enhanced affinity of channel-receptor sites for their respective ligands (see Results). Concomitant with the increase in the number and toxin affinity of STX receptors, we have observed an increase in the sensitivity of Na influx to the inhibitory effect of STX (Fig. 5A). The change in inhibitory potency of TTX during this same period was much less pronounced (Fig. 5B). Such differences between STX and TTX potencies in the same population of sodium channels are not without precedent. In puffer fish (Kidokoro et al., 1974) and *Taricha* newts (Kao and Fuhrman, 1967), species that synthesize and sequester TTX, nerves and muscles have action potentials that are highly resistant to TTX but show almost normal sensitivity to STX. The contrasting situation has been observed in certain bivalve molluscs, particularly those that survive during the "red tides," intense proliferations of the dinoflagellates that synthesize STX; impulses in nerves of these molluscs show marked resistance to STX but are blocked by concentrations of TTX several orders of magnitude lower (Twarog et al., 1972).

Discriminations between TTX and STX by the channel receptor could occur directly from its intrinsic structure; for example, in frog nerve the potency of STX is doubled, but that of TTX is unaffected when D_2O replaces all the solvent H_2O (Hahin and Strichartz, 1981), and in rat skeletal muscle, when the temperature is raised from 4 to 37°C the affinity of TTX is increased by almost a factor of 4, but that of STX is increased by only 1.5 (Hansen Bay and Strichartz, 1980). These observations show that the bonds formed by the channel receptor with TTX molecules are not identical to those formed with STX molecules, and therefore provide an explanation for the discrimination between these two toxins on a molecular level.

The increase in binding affinity and physiological potency of STX that occurs during development might be attributed, at least in part, to changes in the electrically charged groups near the toxin receptor. Studies of toxin binding (Henderson et al., 1974) and of the inhibition of sodium currents by STX and TTX (Hille et al., 1975a) indicate that negatively charged groups fixed to the membrane near the channel toxin receptor can modulate the apparent affinity of STX and TTX. Similar negatively charged groups have been postulated to explain the effects of variations in divalent cation concentration, ionic strength, or pH on the relationship between the membrane potential and the conductance state of sodium and potassium channels and the concomitant changes in excitability of nerve membranes (Frankenhaeuser and Hodgkin, 1957; Gilbert and Ehrenstein, 1969; Hille et al., 1975b; Hahin and Campbell, 1983). The binding of BTX to sodium channels also depends on the conformational state of the channels (Catterall et al., 1981). Therefore, it is possible that modification of discrete, specific membrane charges at the sodium channel occur during development and that these account in part for the increase in STX affinity, the change in the apparent affinity of BTX, and the appearance of stimulatable action potentials.

Sensitivity of sodium channels to TTX and STX does not occur in all

developing tissues to the same extent. In mammals, skeletal muscles express little TTX sensitivity of their action potentials until birth, and this sensitivity increases with age until 3 wk post-natum (Harris and Marshall, 1973), and is partially dependent on continuous innervation of the muscle for its full expression (Sherman and Catterall, 1982). In culture, mammalian muscle cells do not express a high affinity for TTX (Kidokoro, 1975; Lawrence and Catterall, 1981a). Upon denervation, the TTX and STX sensitivity of impulses in mammalian muscle is reduced (Harris and Thesleff, 1971) because the fraction of channels with high affinity for the toxins falls (Pappone, 1980; Hansen Bay and Strichartz, 1980). Avian skeletal muscle shows different developmental features. Neonatal muscle is fully sensitive to TTX and denervation does not produce TTX-resistant impulses (Harris et al., 1973). The results of the present study show that in culture avian muscle cells express a toxin-resistant channel preceding the eventual complement of fully toxin-sensitive channels. Thus, sodium channels resistant to TTX and STX are common to both mammalian and avian embryonic tissues.

Since the cells used in this study were dissociated from the embryonic muscle by trypsin digestion, and since trypsin has been reported to reduce the TTX sensitivity of some excitable cells (Lee et al., 1977; Chemeris et al., 1980), it is possible that the absence of high-affinity receptors in newly fused muscle cells results from this enzyme treatment. However, this possibility is unlikely since our earliest measurements were conducted 48 h after the mild trypsin treatment, and since myoblasts typically undergo two cycles of cell division before our initial measurements, the majority of cells assayed have not been exposed to trypsin.

In a recent study by Frelin et al. (1981), no changes in toxin affinities during development of chick skeletal muscle in culture have been reported. The discrepancy between these findings and our present observations may be accounted for by lack of sufficient synchrony in fusion and muscle differentiation under the culture conditions used by Frelin et al. In addition, it is possible that the developmental changes observed in the present study were obscured in the experiments reported by Frelin et al. because of the use of TTX derivatives. As shown in Fig. 5 and discussed in the preceding paragraphs, the developmental changes are much more readily detected when STX is used rather than TTX.

STX binding and Na⁺ influx activities diminish more rapidly in 3-d cells than in 7-d cells when protein synthesis is blocked (Fig. 6). If we assume that the fall in activities measured after 1 d represents an exponential process, then the half-time for these activities in 3-d cells is 32 and 15 h, whereas extrapolated decreases in 7-d cells imply 67 and 170 h, respectively, for Na influx and STX binding (Table IV). These turnover values probably provide an underestimate of the degradation rate of channel components, because of the continuing appearance on the surface of channel components synthesized before the addition of the protein synthesis inhibitor. The presence of such an intracellular channel precursor pool has been indicated recently by the resolution of kinetics of decline of Na influx during inhibition of protein

synthesis in 5-d cells (Bar-Sagi and Prives, 1983). A period of 5 h passed after CHX addition before the flux activity began to decline exponentially with a half-time of 18 h, a value in the range that we measure for 3-d cells. Theoretically, a sufficient pool of channel precursor proteins could exist in 7-d cells so that after the cessation of protein synthesis, channel activities would not disappear from the surface for a long time, greatly reducing the apparent turnover rate calculated from a single time point.

The differential appearance of Na⁺ channel activities observed in the present study might represent the sequential expression of two distinct channel populations, STX-resistant channels being followed by STX-sensitive channels, or the increase in STX sensitivity may result from functional modification of pre-existing STX-insensitive Na⁺ channels. Such modification could involve post-translational steps such as phosphorylation, glycosylation, selective proteolytic modification, and subunit aggregation. Although the macromolecular events that underlie the developmental appearance of STX sensitivity are as yet unknown, the parallel increases in both metabolic stability of channel components and STX sensitivity may be functionally related. The process that converts channels to the high-affinity STX form may be the same as the one that increases stability, or these two changes may follow in parallel from a common precursor step.

As a final speculation, we venture that the aspects of sodium channels that confer their high affinity for STX and TTX, and are most probably only superficially related to the channel's gating and ion selectivity functions (Spalding, 1980; Huang et al., 1979), are secondary consequences of the structural modifications that subserve spatial and metabolic stability. Some adaptive organisms (e.g., pufferfish, *Taricha* salamanders, frogs, octopus) have been able to exploit these structures in the evolution of specific and highly potent neurotoxins, whereas others (e.g., *Gonyaulax*, the biosynthesizers of STX) have been the more passive beneficiaries of evolving neuronal structures.

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