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

I. Binding Studies¹

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Abstract

[¹²⁵I]Scorpion toxin and [³H]saxitoxin, two neurotoxins that bind specifically to different sites on sodium channels, were used to monitor the development of sodium channels during synchronous differentiation of chick skeletal muscle in culture. [³H]Saxitoxin-binding sites are not detectable in myoblasts. They appear only after myoblasts fuse to form myotubes and increase gradually to reach a maximal value of 58 fmol/mg of protein by 8 days in culture. In contrast, [¹²⁵I]scorpion toxin-binding sites are first detected during the period of cell fusion and increase after fusion to reach a maximum by 4 days in culture, well before either the maximal value of the [³H]saxitoxin binding or the appearance of fully developed sodium action potentials. Neither [³H]saxitoxin binding nor [¹²⁵I]scorpion toxin binding affinity changes during development; they have values of 1.4 and 62 nM, respectively. These data, and data described in the companion paper (Baumgold, J., J. B. Parent, and I. Spector (1983) *J. Neurosci.* 3: 1004-1013), suggest that sodium channel development involves two sequential steps: incorporation into the cell membrane of an immature form of the sodium channel capable of binding scorpion toxin alone, followed by the acquisition of the ability to bind saxitoxin and thus to become a mature and functional channel.

The recent molecular picture of the sodium channel in nerve and muscle postulates the existence of three separate sites for the action of various neurotoxins known to specifically affect this channel (Catterall, 1980a, 1981). Tetrodotoxin (TTX) and saxitoxin (STX) bind to one site and block the inward sodium current responsible for the rising phase of the fast action potential (Narahashi et al., 1964, 1967; Ritchie and Rogart, 1977). Alkaloid toxins such as batrachotoxin, veratridine, and aconitine bind to a second site and induce steady depolarization by modifying various properties of sodium channels (e.g., see Albuquerque, 1972; Khodorov and Revenko, 1979). Finally, polypeptide toxins such as some scorpion toxins (ScTX) and sea anemone toxins bind to a third site and

prolong the action potential primarily by modifying the process of sodium current inactivation (Okamoto et al., 1977; Rathmayer, 1979; Rochat et al., 1979; Gillespie and Meves, 1980). The alkaloid toxin-binding sites and the polypeptide toxin-binding sites may be allosterically coupled (e.g., see Catterall, 1980a; Lazdunski et al., 1980), whereas no interactions have yet been demonstrated between the TTX (or STX)-binding site and the other two sites.

Although the pharmacological properties of each of these three binding sites have been studied extensively (recently reviewed by Ritchie, 1979; Catterall, 1980a, 1981; Lazdunski et al., 1980), it is still unclear how these three sites interact, on a molecular level, to form a functional channel. For example, a variety of nonexcitable cells, including glial cells and fibroblast cell lines, exhibit veratridine-stimulated, TTX-sensitive ²²Na uptake responses (Munson et al., 1979; Romey et al., 1979; Pouyssegur et al., 1980; Frelin et al., 1982) that are clearly consistent with the existence of sodium channels; yet, electrophysiologically, these cells are inexcitable. It appears, therefore, that the sodium channels present in these cells are in an electrophysiologically nonfunctional

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or "silent" form (Romey et al., 1979; Pouyssegur et al., 1980). Furthermore, a number of excitable tissues exhibit normal action potentials that are "insensitive" to TTX (Harris and Thesleff, 1971; Twarog et al., 1972; Harris and Marshall, 1973; Yoshida et al., 1978; Fukuda and Kameyama, 1980; Pappone, 1980). These findings suggest that the functional properties of sodium channels are not simply determined by the presence of the various binding sites.

One approach to the study of the interactions among the various binding sites of this channel involves the use of a system in which the expression of the sodium channel can be examined by biochemical and electrophysiological techniques during the process of differentiation. Chick skeletal muscle grown in culture shows rapid and synchronous differentiation kinetics and is highly suitable for such studies. Previous electrophysiological work using this system has shown that the appearance of sodium action potentials occurs shortly after fusion of myoblasts and that during subsequent development the cultures acquire the ability to generate and propagate fully developed sodium action potentials (Spector and Prives, 1977).

In the present work, we have studied the developmental sequence of the major components of the sodium channel using radiolabeled toxins, and we have related this development to changes in functional properties of these channels assayed by ion influx and electrophysiological techniques. In this paper we present data using radiolabeled STX and ScTX. We show that the binding sites for these two toxins appear at different times during differentiation and that their levels increase at different rates. In the following paper (Baumgold et al., 1983), we describe the development of the functional properties of the sodium channel and propose a model to account for our observations. Parts of this work have been presented in preliminary form (Baumgold et al., 1981; Parent et al., 1981).

Materials and Methods

Cell cultures. Primary cultures of chick pectoral muscle cells from 12-day-old embryos were prepared as described previously (Paterson and Prives, 1973). To obtain rapid differentiation kinetics, cells were plated into 35-mm culture dishes at a density of 4.2×10^4 cells/cm² and grown for the first 114 hr in 85% Richter's improved Eagle's medium prepared by the Media Supply Unit, National Institutes of Health (Richter et al., 1972), 10% horse serum, and 5% chick embryo extract with 50 IU of penicillin/ml and 50 mg of streptomycin/ml. To minimize fibroblast proliferation the cultures were exposed to fluorodeoxyuridine (4×10^{-5} M) and uridine (10^{-4} M) for a 24-hr period after 90 hr in culture. Upon removal of the fluorodeoxyuracil, the amount of embryo extract in the medium was reduced from 5% to 2%. In order to obtain slower kinetics of differentiation, 2.4×10^4 cells/cm² were plated and grown under the same conditions.

Preparation and purification of [³H]saxitoxin. Saxitoxin was obtained from the Food and Drug Administration and was radioactively labeled by New England Nuclear according to the method described by Ritchie et al. (1976). The toxin was purified by high-voltage electro-

phoresis and stored as aliquots at -70°C until use. The concentration and radiochemical purity (64%) of the toxin were determined as previously described (Baumgold, 1980).

Purification, quantitation, and iodination of scorpion toxin. Crude scorpion venom from *Leiurus quinquestriatus* was obtained from Sigma Chemical Co. and purified on an Amberlite CG-50 ion exchange column essentially as described by Catterall (1976, 1981). The eluate from the column was collected and the protein in each fraction was determined by reading the absorbance of the fractions at 280 nm. The pattern thus obtained is shown in Figure 1 (*solid circles*). The fractions containing the active toxin were identified by their ability to stimulate the uptake of ²²Na into N-18 neuroblastoma cells using the method described by Catterall (1976). As shown by the *open circles* in Figure 1, only fractions 105 to 180 from the ion exchange column were effective in stimulating the uptake of ²²Na above control levels. Representative fractions from those were analyzed by gel electrophoresis using the β -alanine buffer system of Fambrough et al. (1968). These Coomassie blue-stained gels are shown in the *inset* in Figure 1 and indicate that the active fractions contain only one protein band. Furthermore, the density of staining of this protein correlates well with the ²²Na uptake activity, further suggesting that these fractions contain the active toxin. The last gel on the *extreme right* is a gel of whole venom. Fractions 120 to 145 were pooled and used in these experiments. The concentration of toxin was determined by measuring the absorbance at 280 nm, multiplying this value by 19% (since bovine serum albumin (BSA) contains 19 tyrosine residues and ScTX contains 5 (Kopeyan et al., 1978), BSA and ScTX each contain 2 tryptophan residues) and determining the concentration of BSA (on a molar basis) with an equivalent absorbance. The purified toxin was iodinated using the lactoperoxidase method (Catterall, 1977; Rochat et al., 1977). Ten nanomoles of dry toxin were redissolved in 25 μ l of 40 mM sodium phosphate buffer, pH 7.0, containing 0.4 mM NaI (10 nmol) and 1 mCi (0.45 nmol) of Na¹²⁵I (from New England Nuclear). To this solution, 3 μ l of 250 μ g/ml of lactoperoxidase (Sigma) and 3 μ l of 6 mM H₂O₂ were added approximately every 15 min. After 1½ hr, the reaction was stopped by adding 200 μ l of 10 mM ammonium acetate, pH 7.0, and the mixture was applied to another Amberlite CG-50 column (0.9 $\times$ 12 cm) equilibrated in 10 mM ammonium acetate, pH 8.5, 1 mg/ml of BSA. The column was first eluted with 10 mM ammonium acetate, pH 8.5, 1 mg/ml of BSA, then with 75 mM ammonium acetate, pH 8.5, 1 mg/ml of BSA, and finally with a linear gradient of from 75 to 150 mM ammonium acetate, pH 8.5, 1 mg/ml of BSA. As shown in Figure 2, only the last peak of radioactivity which came off the column was active in stimulating ²²Na uptake in N-18 neuroblastoma cells. Fractions 35 to 45 were pooled and used in these experiments.

In order to determine the purity of the toxin, a sample was run on a β -alanine gel, the gel was sliced, and each slice was counted in a gamma counter. The results are shown in Figure 3 and indicate that the toxin was substantially pure. The concentration of the radioactive scorpion toxin was estimated by determining the dose-

Figure 1. Purification of scorpion toxin. After pelleting the insoluble components, scorpion toxin was applied to a 0.9×20 cm column of Amberlite CG-50 which had been equilibrated with 10 mM ammonium acetate, pH 7.0. The column was eluted with 20 ml of 10 mM ammonium acetate, pH 7.0, followed by 40 ml of 150 mM ammonium acetate and, finally, by a linear gradient of 150 to 250 mM ammonium acetate, pH 7.0. The absorbance at 280 nm of representative fractions was determined (solid circles). The open circles show the stimulation by representative fractions of ²²Na uptake in N-18 neuroblastoma cells as described under "Materials and Methods." The inset shows the electrophoretic gels of representative fractions having a significant stimulation of ²²Na uptake. These gels were run in the β -alanine buffer system described under "Materials and Methods" and were stained with Coomassie blue. The last gel shows the unpurified scorpion venom.

response relationship for the stimulation of ²²Na uptake in N-18 neuroblastoma cells in the presence of 20 μ M veratridine. The half-maximal concentration, known to be approximately 2 nM in the presence of 20 μ M veratridine (Catterall, 1976; J. Baumgold, unpublished observations), thus provided a way of estimating the concentration of the labeled toxin.

Binding studies. [³H]Saxitoxin binding was determined as follows. The growth medium was aspirated from the culture dish and replaced with a solution of [³H]STX in Eagle's Minimum Essential Medium (MEM) containing 25 mM HEPES. This [³H]STX solution was made immediately before use from frozen aliquots of [³H]STX in order to prevent breakdown of [³H]STX. The cultures were incubated for 30 min at 37°C. The excess toxin was washed off rapidly five times (washing time was less than 10 sec) using cold (2°C) wash medium (same as above) containing 1 mg/ml of BSA. The cells were harvested in 0.5 ml of 0.4 N NaOH and 1 ml of 1% sodium dodecyl sulfate, and the radioactivity was counted using a Beckman liquid scintillation counter

after neutralizing the NaOH with HCl and adding Ultrafluor scintillation cocktail. This procedure was used to determine the total [³H]STX binding. Nonspecific binding was determined by incubating replicate plates with 1 μ M TTX (Calbiochem) in the same solutions as that described above. Specific binding was determined by subtracting the nonspecific binding from the total binding. Binding of [³H]STX to the plastic culture dishes was negligible.

[¹²⁵I]Scorpion toxin binding was determined by exposing the cells to a solution of [¹²⁵I]ScTX in Eagle's MEM containing 25 mM HEPES. The cultures were then incubated for 45 min at 37°C and washed five times using the same wash solution as described above without BSA. The total washing time was 2 to 3 min. The cells were harvested in 0.4 N NaOH and counted in a gamma counter. Nonspecific binding was determined by incubating the cells in the above solutions with an excess (1 to 2 μ M) of unlabeled scorpion toxin. [¹²⁵I]ScTX binding to the plastic culture dishes was negligible. Specific binding was determined by subtracting the amount of [¹²⁵I]ScTX

Figure 2. Purification of [125 I]scorpion toxin. Pure ScTX was labeled with Na 125 I and lactoperoxidase as described under "Materials and Methods." The mixture was then loaded onto a 0.9×12 cm Amberlite CG-50 column equilibrated with 10 mM ammonium acetate, pH 8.5, containing 1 mg/ml of BSA, and the column was eluted first with 10 mM ammonium acetate, pH 8.5, containing 1 mg/ml of BSA, then 75 mM ammonium acetate, pH 8.5, 1 mg/ml of BSA, and, finally, with a linear gradient of from 75 to 150 mM ammonium acetate, pH 8.5, 1 mg/ml of BSA. Fractions of 32 drops each were collected and the radioactivity in an aliquot was determined (solid circles). Representative fractions were tested for their ability to stimulate the uptake of 22 Na in N-18 neuroblastoma cells (open circles).

Figure 3. Electrophoretic profile of [125 I]ScTX. A sample of [125 I]ScTX was loaded onto a polyacrylamide gel run using the β -alanine buffer system described under "Materials and Methods." The gel was sliced and each slice was counted in a gamma counter.

bound in the presence of excess ($1 \mu\text{M}$) unlabeled ScTX from that bound in the absence of excess toxin.

α -[125 I]Bungarotoxin binding was determined by incubating the cells for 45 min (37°C) with 3×10^{-8} M α -[125 I]bungarotoxin (New England Nuclear) in Eagle's MEM containing 25 mM HEPES. Nonspecific binding was determined by incubating the cells with 10^{-5} M decamethonium for 20 min followed by a 45-min incubation with 3×10^{-8} M α -[125 I]bungarotoxin and 10^{-5} M decamethonium.

Total cell protein was determined by the method of Lowry et al. (1951) using BSA as a standard after the cells were harvested and dissolved in 0.4 N NaOH.

Results

Under the culture conditions used, the onset of fusion of mononucleated myoblasts to form multinucleated myotubes occurred about 30 hr after plating and reached a maximal value by about 50 hr (Fig. 8). This very rapid and synchronous burst of cell fusion was followed by a period of very rapid cell growth. By 72 hr, the cultures consisted predominantly of large, flat myotubes. With further time in culture, however, these myotubes did not become cross-striated and only occasionally exhibited spontaneous twitches (e.g., see Spector and Prives, 1977), although they developed full-sized Na $^+$ action potentials. This differentiation scheme is also reflected in the elab-

oration of the acetylcholine receptor (AChR) as assayed by specific α -[125 I]bungarotoxin binding. In accordance with previous work (Spector and Prives, 1977), the AChR appeared shortly after fusion and reached maximal levels (240 fmol/mg of protein) after about 100 hr in culture (Fig. 8). However, AChR levels did not exhibit any gradual decline after this stage, probably due to the lack of spontaneous electrical activity and fast contractile activity (Spector and Prives, 1977). Although the rapid differentiation kinetics used in these studies did not result in full maturation of the electrophysiological and contractile activity, the cells developed large resting potentials of -60 to -70 mV by day 3 in culture, which remained constant during subsequent differentiation. Since ScTX binding has been described as being voltage dependent (Catterall, 1979), this allowed us to eliminate any effect of membrane potential in measuring the number of toxin receptor sites. These culture conditions also enabled us to study the initial events associated with the appearance of STX- and ScTX-binding sites.

Saxitoxin binding. The specific binding of [3 H]STX to 11-day-old cultures was found to be saturable (Fig. 4). The ratio of nonspecific to total binding was 51% at 2 nM [3 H]STX. This rather high ratio of nonspecific to total binding reflects the relatively low binding capacity of these cultures and the possibility that some [3 H]STX remained trapped after the cultures were washed. However, more extensive washing (up to 10 times in 40 sec) did not significantly alter the amount of either nonspecifically bound or specifically bound [3 H]STX. Although detailed studies of the kinetics of [3 H]STX binding were not performed, the rate of [3 H]STX desorption (the "off rate") was found to be slow enough that little or no nonspecifically bound [3 H]STX was desorbed with the washing procedure used.

The dissociation constant (K_d) of binding of [3 H]STX

to 11-day-old cultures was found to be 1.4 nM (Fig. 5), a value that agrees well with the K_d for [3 H]STX binding to a variety of other excitable tissues (Ritchie and Rogart, 1977). The binding curves for 5-day-old cultures are also shown as a double reciprocal plot in Figure 5. The same x -intercept of both lines shows that the K_d of binding does not change during development.

Scorpion toxin binding. The specific binding of [125 I]ScTX to 8-day-old cultures of muscle cells is shown in Figure 6. These cultures bind [125 I]ScTX in a specific and saturable manner. The double reciprocal plot of the binding (Fig. 7) shows a K_d of 62 ± 20 nM. Preliminary experiments indicate that, as with [3 H]STX binding, the K_d does not change with development, although the total amount of binding increases dramatically.

Development of [3 H]STX and [125 I]ScTX binding sites. Figure 8 summarizes the developmental sequence of [3 H]STX and [125 I]ScTX binding sites. [125 I]ScTX binding was detectable before and during the rapid burst of cell fusion. This binding increased sharply after fusion and reached maximal values by 4½ days in culture.

In contrast, no [3 H]STX binding was detected either in myoblasts or during cell fusion. This binding was first detected in newly fused myotubes and increased gradually over the course of several days. It reached its maximal value of 58 fmol/mg of protein by day 8.

The time course of [3 H]STX binding was not influenced by the initial trypsinization treatment used in the normal cell culturing procedure. Cells that were mechanically dissociated without prior trypsinization showed the same amount of [3 H]STX binding as trypsinized cells.

When the initial plating density was decreased to 2.6×10^4 cells/cm 2 , the whole developmental sequence was found to slow down. The major burst of cell fusion was prolonged and delayed by about 12 hr and the period of cell growth was also prolonged. Despite these differences

Figure 4. Binding of [3 H]STX to 11-day-old muscle cultures. The cells were exposed to the indicated concentration of [3 H]STX and incubated for 30 min at 37°C, and the excess toxin was washed off. The cells were then harvested and counted as described under "Materials and Methods." The nonspecific binding was determined by adding 1 μ M TTX to the incubation medium.

Figure 5. Double reciprocal plot of the binding of [^3H]STX to 5-day-old cultures (solid circles) and 11-day-old cultures (open circles). K_d , ~ 1.4 nM.

Figure 6. [^{125}I]ScTX binding to 8-day-old muscle cultures. The cells were exposed to the indicated concentrations of [^{125}I]ScTX and incubated for 45 min at 37°C , and the excess toxin was washed off. The cells were then harvested and counted. The nonspecific binding was determined in the presence of $1\ \mu\text{M}$ unlabeled ScTX.

the [^{125}I]ScTX-binding sites developed first and reached their maximal value before the [^3H]STX-binding sites.

Discussion

It is clear from the data in Figure 8 that the binding sites for [^{125}I]ScTX and for [^3H]STX appear at different times during muscle development and increase with different kinetics. At least two possible interpretations of these results can be made. (1) The two binding sites are

located on different subunits of the sodium channel, each of which is subject to separate biological control; (2) the two binding sites are located on the same subunit (or subunits) which becomes incorporated into the plasma membrane in an immature form, capable of binding ScTX alone, then undergoes structural change enabling it to also bind STX. Although our data do not favor one particular interpretation, they demonstrate that the [^3H]STX-binding sites develop at a very different rate from

the [125 I]ScTX-binding sites and strongly indicate differences in the number and structure of subunits between immature and mature muscle.

Although the subunit composition of sodium channels from chick skeletal muscle has not yet been determined, recent work on the purification of sodium channels from rat brain has shown that it is composed of three subunits: one large subunit of 270,000 daltons and two smaller subunits of 37,000 and 39,000 daltons (Catterall, 1982). Using a photoaffinity derivative of [125 I]ScTX, Beneski and Catterall (1980) have shown that only the 270,000- and the 39,000-dalton subunits bind [125 I]ScTX. It is still

unclear which of the rat brain subunits binds [3 H]STX, but in electric eel tissue, the [3 H]STX receptor is composed of only one 270,000-dalton protein subunit, suggesting that the large subunit may be sufficient for [3 H]STX binding. The [3 H]STX receptor from rat skeletal muscle, like that from brain, has been shown to be composed of three subunits: a large subunit of 125,000 to 250,000 daltons, and two smaller subunits of 47,000 and 37,000 daltons (Barchi, 1983).

The dissociation constant for [125 I]ScTX binding to muscle cells was found to be 62 ± 20 nM (see Fig. 7). In contrast, the dissociation constant for [125 I]ScTX binding

Figure 7. Double reciprocal plot of the binding of [125 I]ScTX to 8-day-old cultures.

Figure 8. Development of [3 H]STX binding (O), [125 I]ScTX binding (●), and α -[125 I]bungarotoxin binding (□) during differentiation of chick skeletal muscle in culture. The dishes were seeded at an initial density of 4.2×10^4 cells/cm 2 . All of the determinations were done on replicate plates and each determination was done in triplicate. The error bars represent $\pm$ SD. Total cell protein was 405 μ g/culture in 4½- to 10-day-old cultures. The percentage of fusion (x) was determined as described by Paterson and Prives (1973).

to NIE-115 neuroblastoma cells was found to be 1 to 3 nM (J. Baumgold and I. Spector, manuscript in preparation). The difference between these two values cannot be attributed to the differences in the resting membrane potentials between these two cell types since the muscle cells have a larger resting membrane potential (-60 to -70 mV; see Baumgold et al., 1983) than the neuroblastoma cells (-50 to -60 mV; I. Spector and J. Baumgold, manuscript in preparation). According to Catterall (1977), the larger the membrane potential across a cell membrane, the lower the dissociation constant for [125 I] ScTX binding (see also Catterall, 1979; Couraud et al., 1980). Since the K_d for [3 H]STX binding is virtually the same in nerve and in muscle (e.g., see Ritchie and Rogart, 1977) but the K_d for [125 I]ScTX binding differs substantially, the sodium channels from nerve and muscle must contain at least one molecular region that differs enough to reflect the different dissociation constants for ScTX binding, yet they must have other regions that have a similar enough molecular structure to reflect the similar dissociation constant for [3 H]STX binding.

The half-maximal effect of ScTX in stimulating the uptake of 22 Na into chick muscle fibers has been shown to be 30 nM (Catterall, 1980b). The 2-fold difference between this value and the dissociation constant for [125 I] ScTX binding to chick muscle found in the present study ($K_d = 62 \pm 20$ nM) may well be attributable to the effect of Na^+ ions in the incubation medium: for the uptake studies, the cells were incubated in a sodium-free medium, whereas the binding studies reported in this work were done in a sodium-containing medium to be able to compare these results with electrophysiological studies (see Baumgold et al., 1983). Jover et al. (1980) have shown that [125 I]ScTX binding to rat brain synaptosomes is sensitive to Na^+ ions in the binding medium. A similar conclusion was reached by Okamoto (1980) in electric eel membranes.

Recently, Frelin et al. (1981) described the appearance of sodium channels in differentiating embryonic chick skeletal muscle using a radiolabeled derivative of TTX. In agreement with the present work, Frelin and co-workers found no detectable toxin binding to myoblasts. However, these authors found that the development of TTX binding closely followed the process of fusion. This may well have been due to the slow kinetics of differentiation and lack of synchrony in their cell culture system.

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