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The *Conus* Toxin Geographutoxin II Distinguishes Two Functional Sodium Channel Subtypes in Rat Muscle Cells Developing *in vitro*

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Sodium currents in cultured rat muscle cells converted to myoballs by treatment with colchicine were recorded using a giga-ohm seal voltage-clamp procedure in the whole-cell configuration. Geographutoxin II (GTX II), a novel polypeptide toxin from the piscivorous marine snail *Conus geographus*, reduces sodium currents in rat myoballs without marked alteration of the time course or voltage dependence of activation of the remaining current. Titration of the inhibition of sodium currents by GTX II showed that, in individual myoballs, a fraction of the sodium current averaging 49 ± 9% (SEM) was inhibited by saturating (25 μM) concentrations of GTX II. The concentration–effect curve fit a noncooperative, 1:1 binding isotherm with a single K_D for GTX II of 19 nM characteristic of inhibition of the TTX-sensitive sodium channels of adult rat muscle. Titration of the sodium current remaining in the presence of 2.5 μM GTX II with TTX gave complete inhibition. The dose–response curve fit a noncooperative, 1:1 binding isotherm with a single K_D for TTX of 1.3 μM characteristic of TTX-insensitive sodium channels of embryonic muscle. The action of GTX II was not frequency dependent. The all-or-none inhibition of these 2 sodium channel subtypes by GTX II suggests substantial structural differences in the region of neurotoxin receptor site 1 on TTX-sensitive and -insensitive sodium channels and provides definitive evidence that these 2 sodium channel subtypes function in parallel in muscle cells developing in the absence of innervation.

Two pharmacologically distinct subtypes of voltage-sensitive sodium channels have been described in mammalian muscle cells. The TTX-sensitive sodium channels of adult muscle are blocked by TTX binding at neurotoxin receptor site 1 with an apparent K_D of approximately 10–20 nM (reviewed by Ritchie and Rogart, 1977). Denervation of adult muscle causes appearance of TTX-insensitive sodium channels with apparent K_D values of approximately 1 μM for TTX (Harris and Thesleff, 1971; Pappone, 1980). TTX-insensitive sodium channels are also present in fetal rat muscle developing *in vivo* (Harris and Marshall, 1973). Dissociated cultures of rat muscle cells developing *in vitro* express both TTX-insensitive sodium channels (Kidokoro et al., 1975; Catterall, 1976; Sastre and Podleski, 1976; Stallcup and Cohn, 1976) and TTX-sensitive sodium channels (Frelin et al., 1983; Sherman et al., 1983), which function in parallel as assessed by ion flux (Sherman et al., 1983), voltage-clamp (Gonoi et al., 1985), and single-channel recording (Weiss and Horn, 1986) methods.

Venom of the marine snail *Conus geographus* contains polypeptide toxins of novel structure (Nakamura et al., 1983; Sato et al., 1983; Cruz et al., 1985) that inhibit skeletal muscle contraction (Nakamura et al., 1983) by preferentially blocking muscle sodium channels (Minoshima et al., 1984; Cruz et al., 1985). Sodium channels in neuronal preparations are not blocked at similar toxin concentrations (Cruz et al., 1985; Ohizumi et al., 1986a). Geographutoxin II (GTX II), the most potent of this family of conotoxins, competitively inhibits binding of ^{3}H-saxitoxin to neurotoxin receptor site 1 on muscle sodium channels at concentrations similar to those that inhibit sodium channel function (Moczydlowski et al., 1986; Ohizumi et al., 1986b; Yanagawa et al., 1986). Saxitoxin binding to sodium channels in synaptosomes or superior cervical ganglion is unaffected at similar concentrations. Since saxitoxin and TTX bind similarly to sodium channels of nerve and adult skeletal muscle, GTX II is the first ligand that distinguishes between the structures of neurotoxin receptor 1 on sodium channels in these tissues. This toxin may therefore provide the most sensitive probe of structural differences in this site on sodium channel subtypes. In these experiments, we have examined the action of this toxin on TTX-sensitive and TTX-insensitive sodium channels in spherical myoballs (Fukuda et al., 1976) prepared from cultured rat muscle cells developing *in vitro* using the giga-ohm seal, whole-cell voltage-clamp procedure of Hamill et al. (1981) as described previously (Gonoi et al., 1985).

Materials and Methods

Materials. Geographutoxin II was purified as described previously (Sato et al., 1983) from venom of the marine snail *C. geographus*. The other materials were obtained from the following sources: TTX and calf skin collagen, Calbiochem; tetraethylammonium (TEA) chloride and colchicine, Sigma Chemical Co.; horse serum and newborn calf serum, KC Biological Inc.; and Dulbecco-Vogt modified Eagle's essential medium (DMEM), Grand Island Biological Co.

Primary cell culture. Skeletal muscle cells were obtained from the forelimbs of 20-d-old embryonic rats and prepared as described previously (Lawrence and Catterall, 1981a). Cells were seeded at a density of 2×10^5 cells/35 mm plastic dish (Falcon) coated with calf skin collagen. The culture medium consisted of 10% horse serum, 5% newborn calf serum, and 85% DMEM. The medium was changed every 2 d. After day 4, the culture medium was supplemented with 0.1 μM colchicine, resulting in formation of myoballs within 24 hr.

Voltage-clamp recordings. Voltage-clamp experiments were performed on myoballs formed from rat muscle cells maintained in culture for 11–13 d instead of 5–10 d as in the previous work (Gonoi et al., 1985). The culture medium was replaced with recording medium in

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which the concentration of Na^+ ions was reduced to 35 mM by replacement with TEA (120 mM TEA-chloride, 30 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose, 5 mM HEPES adjusted to pH 7.4 with NaOH). This solution was used to reduce Na^+ current and thereby avoid series resistance artifacts resulting from the large sodium current in myoballs (Gonoi et al., 1985). Recordings were made at room temperature (22–23°C) on myoballs with diameters of 18–24 μm, smaller than those used in the previous work (Gonoi et al., 1985).

The voltage clamp was based on the 1-pipette giga-ohm seal whole-cell recording technique (Hamill et al., 1981). The EPC-7 List clamp circuit was used. Series resistance of pipettes was compensated by an internal feedback circuit. The internal pipette solution consisted of 85 mM CsF, 60 mM CsCl, 10 mM NaF, and 5 mM EGTA. The pH of the internal medium was adjusted to 7.2 with CsOH. K^+ current was efficiently blocked by Cs^+ ions in the pipette. Tip resistances of the pipettes were about 200 kΩ in the bathing solution. Na^+ conductance (g_{Na}) was calculated from peak currents (I_{Na}) by the relation $g_{Na} = I_{Na}/(E - E_{Na})$, where E is membrane potential and E_{Na} is the reversal potential of the Na^+ current measured for each g_{Na} determination.

For measurements of voltage-dependent activation of Na channels, the holding potential of the cell was maintained at −100 mV. The membrane was hyperpolarized in a prepulse to −160 mV for 100 msec to remove inactivation of Na channels and was then depolarized to test pulse potentials from −90 to +60 mV in intervals of +10 mV for 7 msec. The pulses were repeated at 1 sec intervals. The Na^+ currents increased up to 50% within 10 min after making a seal as the pipette solution exchanged with the intracellular fluid. Toxin inhibition measurements were initiated after steady amplitude of Na^+ currents was obtained. GTX II or TTX was dissolved in recording medium at 4-fold higher concentration than desired final concentrations and was added cumulatively to the dish. Care was taken to apply the toxin in multiple aliquots at several points at least 7 mm from the myoball. Usually the inhibition of g_{Na} by the toxins reached equilibrium within 4 min, but 5–10 min was allowed before recording Na^+ currents for determination of g_{Na} at each concentration. The ratio of the maximum Na^+ conductance in the presence of the toxin to that before adding the toxin was plotted for each experiment. In cumulative TTX inhibition experiments in the presence of GTX II, GTX II was also added to the TTX solutions to keep the final concentration of GTX II constant.

Results

The primary structure of GTX II is illustrated in Figure 1. It is a 22 residue polypeptide containing 3 hydroxyproline residues, 3 positively charged arginine residues, and 3 disulfide bonds (Sato et al., 1983). The guanidine moieties of 2 of the arginine residues are likely to occupy similar positions in neurotoxin receptor site 1 of the sodium channel to the guanidine moieties of TTX and saxitoxin. The larger size of GTX II may provide other points of attachment that result in selective binding to sodium channel subtypes.

Rat muscle cells were dissociated from embryonic limb muscles and myoballs were prepared as described under Materials and Methods. A giga-ohm seal was formed on an individual myoball, and sodium currents were recorded over approximately 10 min while the intracellular solution exchanged with the pipette solution and the amplitude of sodium currents reached steady state. A family of sodium current responses elicited by depolarization to membrane potentials of −90 to +60 mV before toxin treatment is illustrated in Figure 2*A*. The sodium channel density and the kinetic and voltage-dependent parameters describing these sodium currents agreed closely with those measured previously (Gonoi et al., 1985). Addition of GTX II to a final concentration of 2.5 μM in the recording medium caused a progressive reduction in the sodium current recorded

Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Arg-Lys-Cys-Lys-

Asp-Arg-Arg-Cys-Lys-Hyp-Met-Lys-Cys-Cys-Ala-NH_2

Figure 1. Primary structure of GTX II.

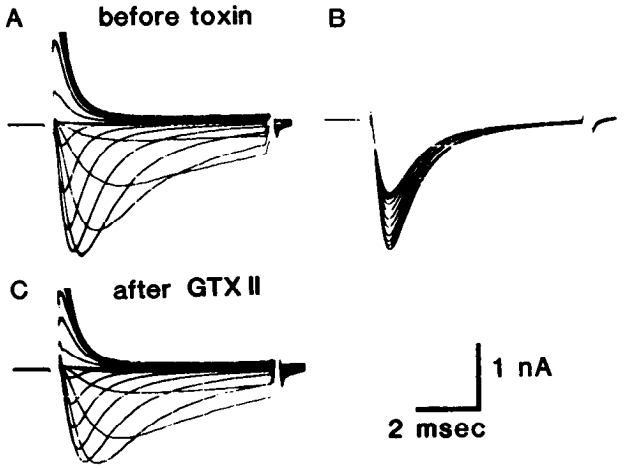

Figure 2. Sodium currents of a rat myoball under voltage-clamp conditions before and after applying GTX II. *A*, Family of sodium currents from myoball in a 13-d-old culture was recorded 10.5 min after making a seal between the myoball and a micropipette from a holding potential of −100 mV as described under Materials and Methods. *B*, GTX II, 167 μl at 10 μM, in recording medium was applied from the outside of the myoball to give a final concentration of the toxin in the bath of 2.5×10^{-6} M. Reduction of the sodium current after applying the toxin was monitored every 10 sec by depolarizing the myoball to a test pulse potential of −20 mV for 7 msec following a prepulse to −160 mV for 100 msec. The photograph presented was taken 3 min after applying GTX II and shows superimposed sodium currents. The peak sodium current decreased with each measurement after applying toxin until a new steady state was attained in 2 min. *C*, Family of sodium currents 5 min after the application of GTX II from the same cell as in *A* and *B*. Stimulus conditions were same as in *A*. Calibration bars are common to *A–C*.

at a pulse potential of −20 mV. The sodium current reached a new steady state after approximately 2 min (Fig. 2*B*). Neither the time course nor the voltage dependence of the remaining sodium current was altered markedly by toxin treatment (Fig. 2*C*).

Cumulative addition of GTX II to individual myoballs under voltage clamp caused progressive reduction in peak sodium conductance as illustrated for 2 representative myoballs in Figure 3. After each addition, sodium conductance declined to a new steady state within 5 min. Currents were recorded within 10 min after each addition. For the 2 myoballs illustrated, 61 and 35% of the sodium conductance were inhibited at a maximum concentration of GTX II. Concentration–effect curves, calculated by least-squares analysis assuming noncooperative 1:1 binding of GTX II to 61 and 35% of the sodium channels, respectively, fit the data closely and yield apparent K_D values 21 and 27 nM for GTX II action on these 2 myoballs. These apparent K_D values agree closely with those for block of contraction, sodium currents, and ^{3}H-saxitoxin binding in adult muscle (Nakamura et al., 1983; Cruz et al., 1985; Ohizumi et al., 1986b) and therefore represent inhibition of TTX-sensitive sodium channels in the rat muscle cells.

In order to analyze the mean properties of a larger number of myoballs, 4 myoballs in different petri dishes were studied at each GTX II concentration and mean values of the ratio of sodium conductance in the presence and absence of GTX II were determined. Figure 4 illustrates the measured sodium conductance as a function of GTX II concentration. Analysis of these data as described above showed that 49 ± 9% of the sodium conductance was inhibited with an apparent K_D of 10

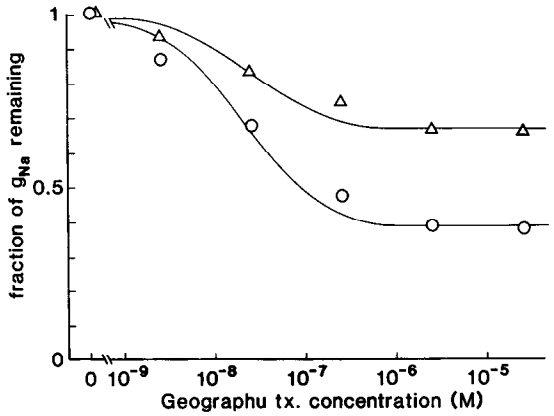

Figure 3. Inhibition of sodium conductance in individual myoballs by GTX II. Sodium currents were recorded from two 12-d-old myoballs as described under Materials and Methods. For each point, GTX II was added from a stock solution of 60 μM in recording medium to achieve the final concentrations indicated. The smooth curves correspond to a least-squares fit of the data assuming a 1:1 binding of GTX II to a fraction (F) of sodium channels: △, $F = 0.35$, $K_D = 21$ nM; ○, $F = 0.61$, $K_D = 27$ μM.

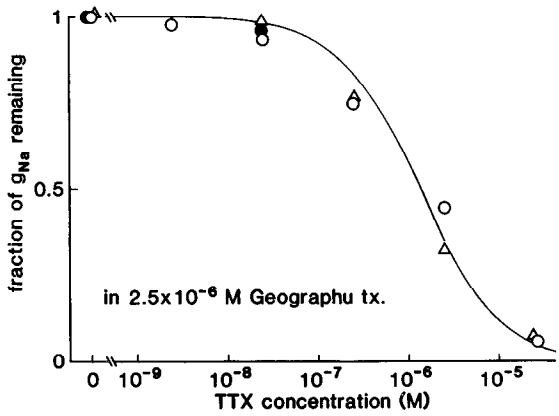

Figure 5. Concentration dependence of inhibition of sodium conductance by TTX in the presence of 2.5 μM GTX II. Myoballs were incubated in the presence of 2.5×10^{-6} M GTX II at room temperature (22–23°C) for 10 min before making a seal with a glass micropipette. After recording sodium currents in the presence of only GTX II, TTX concentration was increased cumulatively while the concentration of GTX II was kept constant and sodium currents were recorded. The different symbols represent different myoballs. The smooth curves in the figure represent a least-squares fit assuming 1:1 noncooperative binding of TTX to Na channels. K_D was 1.3 μM for TTX.

nM, in agreement with the data from studies of individual myoballs.

These results show that individual myoballs contain 2 classes of sodium channels with respect to inhibition by GTX II. The sodium channels that are inhibited by GTX II have the same affinity for GTX II as the TTX-sensitive sodium channels in adult muscle. If TTX-sensitive sodium channels are preferentially inhibited by GTX II, the sodium channels remaining active in the presence of a saturating concentration of GTX II should all be the TTX-insensitive subtype. Figure 5 illustrates a concentration–effect curve for inhibition by TTX of the sodium conductance that remains in the presence of 2.5 μM GTX II. In the cell cultures used for this experiment, 2.5 μM GTX II reduced sodium conductance to 55.8 ± 8.4% (SEM, $n = 6$) of control values. The sodium conductance that remained was unaffected by 25 nM TTX, indicating that no TTX-sensitive sodium channels remained active in the presence of 2.5 μM GTX II (Fig. 5). The remaining sodium conductance was completely inhibited by higher concentrations of TTX with an apparent K_D value of 1.3 μM, identical to that for block of TTX-insensitive sodium channels (Kidokoro et al., 1975; Catterall, 1976; Sastre and Podleski, 1976; Stallcup and Cohn, 1976; Pappone, 1980; Lawrence and Catterall, 1981a; Frelin et al., 1983; Sherman et al., 1983; Gonoi et al., 1985). The concentration–effect curve calculated assuming noncooperative, 1:1 binding to a single site fits the data closely. The results show that GTX II completely blocks the TTX-sensitive sodium channels in cultured rat muscle cells, leaving TTX-insensitive sodium channels unaffected.

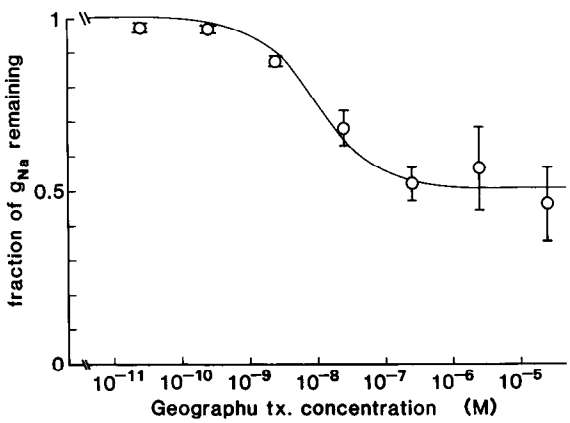

Figure 4. Concentration dependence of inhibition of sodium conductance by GTX II. Sodium currents were recorded from 28 myoballs as described under Materials and Methods. For each myoball, currents were recorded in recording medium and then in recording medium containing the indicated concentration of GTX II. The fraction of sodium conductance remaining in the presence of GTX II was calculated at each toxin concentration and the mean (±SD) was calculated from the results on 4 myoballs at each concentration. The smooth curve was obtained by a least-squares fit of the data assuming a 1:1 binding of GTX II to a fraction (F) of the sodium channels: $F = 0.49$, $K_D = 10$ nM.

Inhibition of TTX-insensitive sodium channels by TTX in heart (Cohen et al., 1981) and skeletal muscle (Gonoi et al., 1985) is frequency dependent, while inhibition of TTX-sensitive channels is not. We examined whether repetitive depolarization enhances block of sodium channels in myoballs by GTX II by stimulating with a train of 20 test pulses from a holding potential of −120 to −20 mV for 10 msec at a frequency of 0.5, 1, or 2 Hz. Similar conditions cause frequency-dependent block of TTX-insensitive sodium channels in myoballs by TTX (Gonoi et al., 1985). In the presence of 25 nM, 2.5 μM, or 25 μM GTX II, no enhancement of sodium channel inhibition by repetitive stimulation was observed. Thus, repetitive activation of sodium channels does not induce block of TTX-insensitive sodium channels by GTX II and the block of TTX-sensitive sodium channels by this agent is not frequency dependent under these conditions.

Discussion

Our results further establish GTX II as the most selective ligand for neurotoxin receptor site 1 on the sodium channel. Not only does this toxin distinguish clearly between TTX-sensitive sodium channels in nerve and muscle (Cruz et al., 1985; Ohizumi et al., 1986a), but it also distinguishes more clearly between the TTX-sensitive and -insensitive sodium channels in rat muscle than does TTX itself. TTX binds to TTX-sensitive sodium

channels with approximately 200-fold higher affinity than TTX-insensitive sodium channels. GTX II binds to and inhibits TTX-sensitive sodium channels with at least 10,000-fold higher affinity than TTX-insensitive sodium channels (Figs. 3, 4). It may have no action on TTX-insensitive sodium channels at all. These results provide the clearest evidence to date that the TTX-sensitive and -insensitive sodium channels in skeletal muscle are structurally distinct entities. TTX-insensitive sodium channels in skeletal muscle have 3 additional properties that distinguish them from TTX-sensitive sodium channels. (1) They have higher affinity for *Anemonia sulcata* sea anemone toxin II than for *Leiurus* α-scorpion toxin at neurotoxin receptor site 3 (Lawrence and Catterall, 1981a, b; Frelin et al., 1984). (2) Their inhibition by TTX is frequency dependent (Gonoi et al., 1985). (3) They have lower single-channel conductance and altered voltage-dependence (Weiss and Horn, 1986). TTX-insensitive sodium channels with similar properties are present in mammalian cardiac cells at all times (Reuter, 1979; Catterall and Coppersmith, 1981; Cohen et al., 1981). Considered together, these observations provide strong evidence that these 2 classes of sodium channels represent distinct pharmacological subtypes that are differentially expressed in muscle tissues.

The present results also provide the clearest evidence to date that rat muscle cells cultured *in vitro* in the absence of neurons are able to synthesize functional TTX-sensitive sodium channels characteristic of adult skeletal muscle. In previous studies (Sherman et al., 1983; Gonoi et al., 1985; Weiss and Horn, 1986), the presence of functional forms of TTX-sensitive and -insensitive sodium channels in cultured muscle cells has been inferred from analysis of biphasic TTX inhibition curves, which revealed 2 components of sodium conductance with apparent K_D values characteristic of these 2 channel subtypes. In contrast, GTX II gives all-or-none inhibition of these 2 channel subtypes, providing a definitive demonstration of the existence of 2 functional classes of channels. Evidently, rat muscle cells have the intrinsic capacity to synthesize functional TTX-sensitive sodium channels in the absence of innervation *in vivo* (Sherman and Catterall, 1982) and *in vitro* (Sherman et al., 1983; Gonoi et al., 1985; Weiss and Horn, 1986; this report). Innervation, and the electrical activity it stimulates in the muscle cell, regulates the cell-surface density of functional channels (Sherman and Catterall, 1982, 1984). GTX II will be a valuable experimental probe in further studies of this regulatory process.

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