0026-895X/00/040692-09$3.00/0
MOLECULAR PHARMACOLOGY
Copyright © 2000 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 58:692–700, 2000

Vol. 58, No. 4
13063/851404
Printed in U.S.A.

An Analysis of the Variations in Potency of Grayanotoxin Analogs in Modifying Frog Sodium Channels of Differing Subtypes

MASUHIDE YAKEHIRO, TSUNETSUGU YUKI, KAORU YAMAOKA, TOSHIAKI FURUE, YASUO MORI, KEIJI IMOTO, and ISSEI SEYAMA

Division of Physiology, Department of Clinical Engineering, Hiroshima International University, Faculty of Health Sciences, Hiroshima, Japan (M.Y.); Department of Physiology, School of Medicine, Hiroshima University, Hiroshima, Japan (T.F., T.Y., K.Y., I.S.); and Department of Information Physiology, National Institute for Physiological Sciences, Okazaki, Japan (Y.M., K.I.)

Received November 2, 1999; accepted June 5, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Responses of tetrodotoxin-sensitive (TTX-s) and insensitive (TTX-i) Na^+ channels, in frog dorsal root ganglion (DRG) cells and frog heart Na^+ channels, to two grayanotoxin (GTX) analogs, GTX-I and α-dihydro-GTX-II, were examined using the patch clamp method. GTX-evoked modification occurred only when repetitive depolarizing pulses preceded a single test depolarization; modification, during the test pulse, was manifested by a decrease in peak Na^+ current accompanied by a sustained Na^+ current. GTX-evoked modification of whole-cell Na^+ currents was quantified by normalizing the conductance for sustained currents through GTX-modified Na^+ channels to that for the peak current through unmodified Na^+ channels. The dose-response relation for GTX-modified Na^+ channels was constructed by plotting the normalized slope conductance against GTX concentration. With respect to DRG TTX-i Na^+ channels, the EC_{50} and maximal normalized slope conductance were estimated to be 31 μM and 0.23, respectively, for GTX-I, and 54 μM and 0.37, respectively, for α-dihydro-GTX-II. By contrast, TTX-s Na^+ channels in DRG cells and Na^+ channels in ventricular myocytes were found to have a much lower sensitivity to both GTX analogs. In single-channel recording on DRG cells and ventricular myocytes, Na^+ channels modified by the two GTX analogs (both at 100 μM), had similar relative conductances (range, 0.25–0.42) and open channel probabilities (range, 0.5–0.71). From these observations, we conclude that the differences in responsiveness of DRG TTX-i, and ventricular whole cell Na^+ currents to the GTX analogs studied are related to the number of Na^+ channels modified.

Since the pioneering work of Numa's group (Noda et al., 1984), a wide variety of cloned sequences of Na^+ channels have been obtained from different organs in the same species as well as from identical organs among different species. As a new family of Na^+ channels, Akopian et al. (1996) have been able to isolate and clone a new type of tetrodotoxin-insensitive (TTX-i) Na^+ channel from dorsal root ganglion (DRG) neurons. In TTX-i Na^+ channels of rat DRG cells, 65% of an open reading frame encoding a 1957-amino-acid protein was shown to be identical with that of the rat cardiac TTX-i Na^+ channel. TTX-i Na^+ channels have been reported to retain unique pharmacological properties that are qualitatively as well as quantitatively different from those of TTX-sensitive (TTX-s) Na^+ channels. It has been shown that versutoxin and robustoxin purified from the venoms of Australian funnel-web spiders have no effect on TTX-i Na^+ channels but produce a dose-dependent slowing or removal of sodium channel inactivation and a reduction in peak I_{Na} in TTX-s Na^+ channels (Nicholson et al., 1994, 1998). TTX-s Na^+ channels are also more susceptible to local anesthetics than TTX-i Na^+ channels (Scholz et al., 1998b): IC_{50} for the tonic block of TTX-i Na^+ channels by lidocaine was 210 μM, whereas TTX-s Na^+ channels showed an IC_{50} value five times lower at 42 μM. On the contrary, Scholz et al. (1998a) have shown that halothane suppresses fast and slow TTX-i Na^+ channels with IC_{50} values of 5.4 and 7.4 mM, whereas it suppresses TTX-s Na^+ channels with a slightly higher IC_{50} value of 12.1 mM. Ethanol at the concentration of 200 mM suppresses maximal available TTX-i Na^+ channels by 18% and TTX-s Na^+ channels by 7% (Wu and Kendig, 1998).

Some of the biological toxins that act selectively on sodium channels have been used extensively as useful tools for analyzing the characteristics of Na^+ channel-gating processes. Batrachotoxin (BTX), grayanotoxin (GTX), veratridine, and aconitine, classified as toxins binding to site 2 (Catterall, 1980), are endowed with some characteristics in common: 1)

This work was supported by grants from the Ministry of Education, Science, and Culture in Japan (to I.S., K.Y., and K.I.).

ABBREVIATIONS: TTX-i, tetrodotoxin-insensitive; DRG, dorsal root ganglion; TTX-s, tetrodotoxin-sensitive; BTX, batrachotoxin; GTX, grayanotoxin; P_o, open channel probability; GHK, Goldman-Hodgkin-Katz.

they bind to the sodium channel in its open state, 2) the modified sodium channel loses the inactivation process, and 3) the activation voltage of the modified sodium channel is shifted in the direction of hyperpolarization (Khodorov, 1985; Narahashi and Herman, 1992).

Thus, it is worth examining how different Na^+ channel isoforms respond to GTX analogs to obtain information on the molecular structure of the gating mechanism of Na^+ channel. In this study, we compared the pharmacological action of GTX analogs on TTX-s and TTX-i Na^+ channels in DRG cells and heart Na^+ channels of the frog, and have thereby obtained novel information about the difference in GTX actions among these Na^+ channel isoforms and the interaction of GTX analogs with their binding sites on the intracellular aspect of Na^+ channels.

Materials and Methods

Cell Preparations. Frogs (*Rana catesbeiana*) were sacrificed by decapitation and the spinal cord was destroyed. Single ventricular myocytes were taken from the hearts using essentially the same technique as described previously (Seyama and Yamaoka, 1988). Briefly, the heart was mounted on a Langendorff apparatus and perfused retrogradely via the aorta with a Ca^{2+}-free solution containing Yakult collagenase (0.025 mg/ml; Yakult, Tokyo, Japan), Wako collagenase (0.35 mg/ml; Wako Pure Chemical Industries Ltd, Osaka, Japan), type III trypsin (0.06 mg/ml; Sigma Ltd., St. Louis, MO), and crystallized BSA (0.6 mg/ml; Seikagaku Corporation, Tokyo, Japan) for 20 min at 32°C. The dispersed cells were kept in a solution containing reduced (200 μM) Ca^{2+} for 30 min and then centrifuged for 1 min at 65*g*. After eliminating the cell debris, the collected cells were maintained in Leibowitz's L-15 medium (Gibco Laboratories, Gaithersburg, MD) until experimental use.

DRG were dissected from bullfrogs. The ganglia were minced and then shaken for 60 to 90 min at room temperature in cell dispersion medium containing 2 mg/ml collagenase (type IA; Sigma), 0.3 mg/ml trypsin (type IX; Sigma) and 0.2 mg/ml deoxyribonuclease I (Sigma). The cell dispersion solution contained 120 mM NaCl, 10 mM glucose, 10 mM HEPES, 2.5 mM KCl, 0.01 mM $CaCl_2$, and pH was adjusted to 7.2 with NaOH. The dispersed cells were centrifuged for 1 min at 65*g*, and the collected cells were stored in modified L-15 medium: 36 ml of 1 mM $CaCl_2$ solution and 7.15 ml of fetal bovine serum were added to 100 ml of L-15 medium (Campbell, 1992).

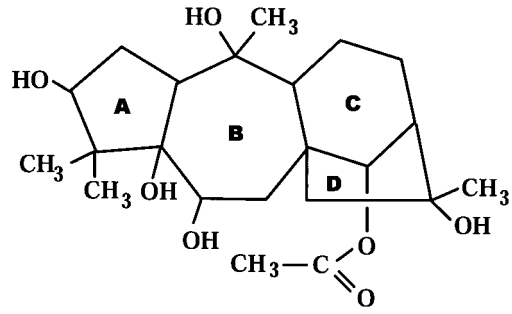
Solutions and Chemicals. For measuring whole-cell Na^+ current, the composition of the external solution was 90 mM NaCl, 15 mM tetraethylammonium chloride, 9 mM $MgCl_2$, 1 mM $CaCl_2$, 0.005 mM $LaCl_3$, and 10 mM HEPES. The pH of the external solution was adjusted to 7.2 with NaOH. The internal solution consisted of 60 mM CsF, 40 mM CsCl, 20 mM NaF, 5 mM EGTA, and 5 mM HEPES. The pH of the internal solution was adjusted to 7.0 with CsOH.

To assess the effects of GTX on whole-cell Na^+ currents, GTX analogs were added to the pipette solution, because GTX is known to act intracellularly (Seyama et al., 1988). Pipette solutions for cell-attached single-channel recordings contained 250 mM NaCl, 0.3 mM $CaCl_2$, 1 mM $MgCl_2{\cdot}6H_2O$, 0.01 mM $LaCl_3{\cdot}7H_2O$, 5 mM HEPES, and 0.001 mM TTX. The pH was adjusted to 7.2 with NaOH. Bath solutions for DRG cells contained 120 mM KCl, 75 mM CsCl, 1 mM $CaCl_2{\cdot}7H_2O$, 2 mM $MgCl_2{\cdot}6H_2O$, and 5 mM HEPES, and that for ventricular myocytes contained 115 mM KCl, 3 mM $MgSO_4{\cdot}7H_2O$, and 5 mM HEPES. The pH of both bath solutions was adjusted to 7.2 with KOH.

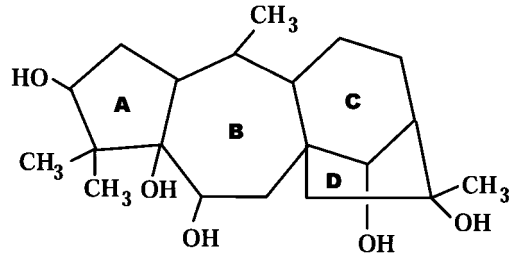
The structural formulae of the GTX analogs used in this study are shown in Fig. 1. Both GTX analogs were kindly provided by Professor Emeritus J. Iwasa of Okayama University, Faculty of Agriculture, Okayama, Japan. All stock solutions of GTX analogs were dissolved in dimethyl sulfoxide at a concentration of 10^{-1} or 10^{-2} M.

Because GTX molecules are permeable through cell membranes and the pipettes containing GTX act as a point source, one may be concerned how evenly GTX was distributed in the intracellular phase and whether the diffusion of GTX out of the membrane to the external phase lowered the concentration of toxin in the internal side. We resolved this concern by showing that GTX-modification occurred to the same extent whether GTX of the same concentration was present on both sides or only on the internal side. To begin with, we collected necessary data for constructing current-voltage curves with and without conditioning pulses using a patch electrode containing 100 μM GTX-I in ventricular myocytes. Successively, GTX-I of the same concentration was additionally introduced into the external side, and the same kinds of data from the same preparation were collected after soaking the preparation for 20 min. The resultant normalized slope conductances (see under *Dose-Response Relationship for GTX-Evoked Modification*) from four series of experiments were estimated to be 0.044 ± 0.008 for internal and 0.047 ± 0.009 for both sides applications (n = 4). Because there was no statistical significance in the difference between the two values, it is reasonable to assume that the cell membranes functioned as an effective diffusion barrier.

Ionic Current Recordings. Whole-cell patch pipettes with a resistance of less than 2 MΩ were used for obtaining optimum voltage control. Whole-cell currents (filtered at 5 KHz) were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Routine series-resistance compensation was performed to values more than 80% to minimize voltage-clamp errors. Recordings were started 5 min after establishing a conventional whole-cell recording configuration. Single Na^+ channel currents were measured using the cell-attached variation of the patch clamp technique (Hamill et al., 1981). Single-channel currents were filtered at 5 KHz except those in Fig. 6. In Fig. 6, 1 KHz filtering was set on recording data and further digital filtering at a cut-off frequency of 400 Hz was employed. The whole-cell membrane currents were digitized at a sampling frequency of 20 to 100 kHz with a 12-bit analog-to-digital converter (TL-1 DMA interface; Axon Instruments), controlled by pClamp software (Axon Instruments); digitized currents were stored



Grayanotoxin I (GTX-I)



α-dihydro-grayanotoxin II (α-dihydro-GTX-II)

Fig. 1. Structure of GTX-I and α-dihydro-GTX-II.

on diskettes. In single-channel recordings, the sampling rate ranged from 5 KHz to 100 KHz. Data are presented as mean ± 1 SD along with the number of observations made (n). In the whole-cell configuration, the background current was subtracted from the total current recorded to obtain the actual Na^+ current. The background current records required were recorded in 0.3 μM TTX solutions for ventricular myocytes (Yamaoka, 1987), and in solutions containing 1.0 μM TTX plus 1 mM Cd^{2+} for DRG cells. All experiments were conducted at room temperature (23–26°C).

Single Channel Analysis. Open channel probability ($P_{o,GTX}$) and dwell time histograms for GTX-modified Na^+ channels (at −60 mV) were obtained from the segment in a record having no overlapping openings for at least 10 s (Fig. 6, A and D). $P_{o,GTX}$ and the histograms were calculated using idealized record analysis (pClamp software).

The procedure for obtaining open channel probability under control conditions (at the time of peak Na^+ current; $P_{o,control}$) was as follows. First, we averaged 100 to 150 traces at −10 mV (multichannel recordings) to get an ensemble record. Next, we inspected these 100 to 150 traces and selected the trace containing the maximum number of simultaneously open channels, referring to maximal individual ionic current. We estimated the number of channels in the patch (N) by dividing the peak of maximal individual ionic current by the single channel current. Separately, we also divided the peak of ensemble current by the single channel current to obtain NP_o. Then, we obtained $P_{o,control}$ by dividing NP_o by N. Therefore, $P_{o,control}$ indicates the open probability of a single channel at the peak of ensemble record. $P_{o,control}$, thus estimated, was found to be 0.40 ± 0.08 (n = 5) for DRG TTX-i Na^+ channels and 0.32 ± 0.06 (n = 5) for ventricular Na^+ channels. In ventricular myocytes, the maximum individual ionic currents used in this calculation were less than 7.4 pA, which corresponds to the summation of four single-channel unit currents. Regarding DRG TTX-i Na^+ channels, the maximum individual ionic current in most cases was less than 4.8 pA, which corresponds to the summation of four single-channel unit currents. The estimated values are thought to approximate to the true value of P_o for a single channel, because a small number of channels within a membrane patch leads to a higher likelihood that all channels in a patch will open simultaneously at least once in a large series of trials. The values of $P_{o,control}$ obtained in this study are in agreement with values reported by Aldrich et al. (1983). Capacitative and leakage currents were subtracted from the records using blank traces without channel openings.

Separation of TTX-i and TTX-s Na^+ Currents in Isolated DRG Cells. DRG cells have been reported to contain two types of Na^+ channels, TTX-s and TTX-i Na^+ channels. Because Hille (1968) has demonstrated that 0.3 μM TTX blocks the Na^+ channels of the frog node of Ranvier, the sensitivity of the Na^+ channels of DRG cells to 0.3 μM TTX was examined. The amplitude of the Na^+ current (under voltage clamp conditions) was decreased to 20 to 70% of the control value. The residual Na^+ current remained unchanged, even when the concentration of TTX was increased to 1.0 μM. This finding indicates that 0.3 μM TTX is sufficient in concentration to completely block TTX-s Na^+ channels. Residual Na^+ current with 0.3 μM TTX treatment can be suppressed by 0.3 mM Cd^{2+} (Narahashi et al., 1994; Akopian et al., 1996). In separate experiments, the IC_{50} value for Cd^{2+} in suppressing the Na^+ current was estimated to be 8.1 ± 2.0 μM (n = 3) from the dose-response curve for Cd^{2+} in the presence of 0.3 μM TTX. To obtain the IC_{50} value for TTX-sensitive Na^+ channel, we increased the external concentration of Cd^{2+} up to 10 mM from 0.3 mM. The resultant dose-response curves yielded an IC_{50} value of 10.0 ± 2.2 mM (n = 7). There is a big difference in the sensitivity to Cd^{2+} between TTX-i and TTX-s Na^+ channels, enough to separate one from the other pharmacologically. From these observations, we define Na^+ channels activated in the presence of 1.0 μM TTX as TTX-i Na^+ channels and those seen in the presence of 0.3 or 1 mM Cd^{2+} as TTX-s Na^+ channel.

Results

Whole-Cell Recordings

Time Course of GTX Modification of Na^+ Channels. As previously reported, a prerequisite for GTX-induced modification of Na^+ channels in squid axons is a sustained depolarization to a membrane potential more positive than −80 mV (Yakehiro et al., 1997). However, in the present experiments, the application of a single depolarizing prepulse was not effective in modifying cardiac or DRG cell Na^+ channels. We therefore examined the time course of GTX-modification and recovery from modification. Figure 2 shows a typical example of the time course of GTX-I (100 μM) modification for ventricular Na^+ channels. Because GTX shifts the activation curve of Na^+ channels to the hyperpolarizing direction, GTX-induced modification was recognized as a sustained inward Na^+ current, in response to the test pulse to −80 mV from a holding potential of −120 mV after repetitive conditioning pulses. As the numbers of conditioning pulses became larger, a sustained inward current flowing at the end

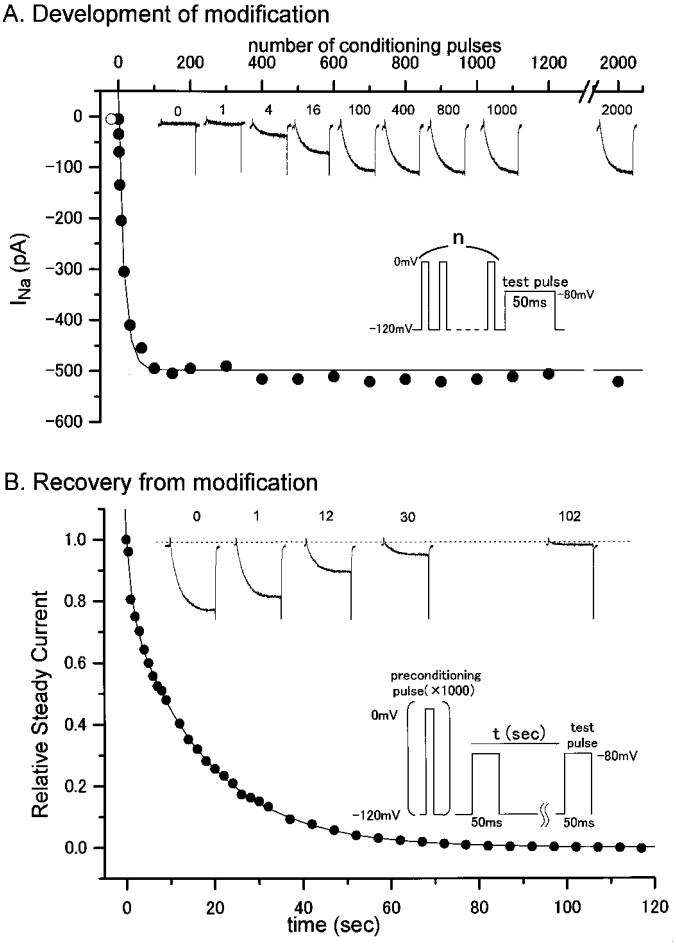


Fig. 2. Time course for development and decay of modified currents through Na^+ channels. The internal solution contained 100 μM GTX-I. A, change in sustained currents after applying conditioning pulses are presented as the function of numbers of conditioning depolarizing pulses (●). Conditioning pulses to 0 mV of 5 ms in duration from holding potential of −120 mV with interpulse interval of 13 ms. ○ at the very beginning of record represents sustained current at 120 s after the termination of conditioning pulses. B, ventricular cells were subjected to 1000 repetitive depolarizing pulses having the same qualities of conditioning pulses as in A. The resultant sustained currents in the response to test pulse to −80 mV were plotted against various time intervals after conditioning pulses.

of the pulse to −80 mV gradually increased. A quick development of sustained Na^+ current soon attained a steady state when the number of conditioning pulses was more than 100. To induce full modification of DRG TTX-s and TTX-i Na^+ channels, approximately 100 repetitive conditioning pulses were also required. Because GTX modification is reversible and recovery from modification is independent of GTX concentration (Yakehiro et al., 1997), the time course of recovery from modification in isolated ventricular myocytes at a membrane potential of −120 mV was determined by plotting sustained currents during the test pulse to −80 mV against various intervals after 1000 (GTX-I) conditioning pulses to 0 mV. The time course of current decay could be described as the sum of two exponentials (Fig. 2B): $\tau_1 = 1.4 \pm 0.5$ s, $\tau_2 = 18.6 \pm 1.8$ s ($n = 3$) for GTX-I and $\tau_1 = 0.5 \pm 0.2$ s, $\tau_2 = 5.4 \pm 0.8$ s ($n = 3$) for α-dihydro-GTX-II. Because the conditioning interpulse interval was much briefer than the time constants, the release of GTX analogs should not substantially affect the rate of GTX-induced modification of Na^+ channels.

GTX-Evoked Modification of Na^+ Channels in DRG Cells and Ventricular Myocytes. After blocking TTX-s Na^+ channels using 1.0 μM TTX, the residual (TTX-i) current was modified by the application of 150 conditioning pulses in the presence of 300 μM α-dihydro-GTX-II. As shown in the middle panel of Fig. 3A, the peak currents recorded from GTX-modified channels are suppressed in amplitude relative to the control current, and sustained currents appear at large negative membrane potentials, thus shifting the activation voltage in the hyperpolarizing direction by as much as 50 mV. After blocking the TTX-i channels by external Cd^{2+} (1 mM), the residual TTX-s Na^+ current was modified similarly by 300 conditioning pulses above in the presence of 300 μM α-dihydro-GTX-II (Fig. 3B). The current-voltage relationship again reveals a shift of the activation curve in the hyperpolarizing direction (Fig. 3B). In isolated ventriculomyocytes, 1000 conditioning pulses from a holding potential of −80 mV modified the cardiac Na^+ current in a manner qualitatively similar to that in the other kinds of Na^+ channels studied (Fig. 3C). In Fig. 3, it is not clear whether GTX-induced modification of Na^+ channels reaches saturation level or not. To obtain more accurate data for modification, we conducted a single-channel experiment as presented in a later section.

Dose-Response Relationship for GTX-Evoked Modification. Because the number of Na^+ channels expressed on a cell varies, it is necessary for GTX-modified Na^+ channel to be normalized to a representative factor of normal Na^+ channel in the active state. We chose the maximum permeability constant of the Goldman-Hodgkin-Katz (GHK) equation (Goldman, 1943; Hodgkin and Katz, 1949) for this purpose, because the current-voltage relationship for Na^+ channels, particularly modified ones, show a concave curve. The degree of GTX-evoked modification of Na^+ channels at various concentrations of GTX in the pipette was estimated as the permeability constant for sustained (i.e., modified) Na^+ currents after conditioning prepulses, referred to the maximum permeability constant for the unmodified peak Na^+ current

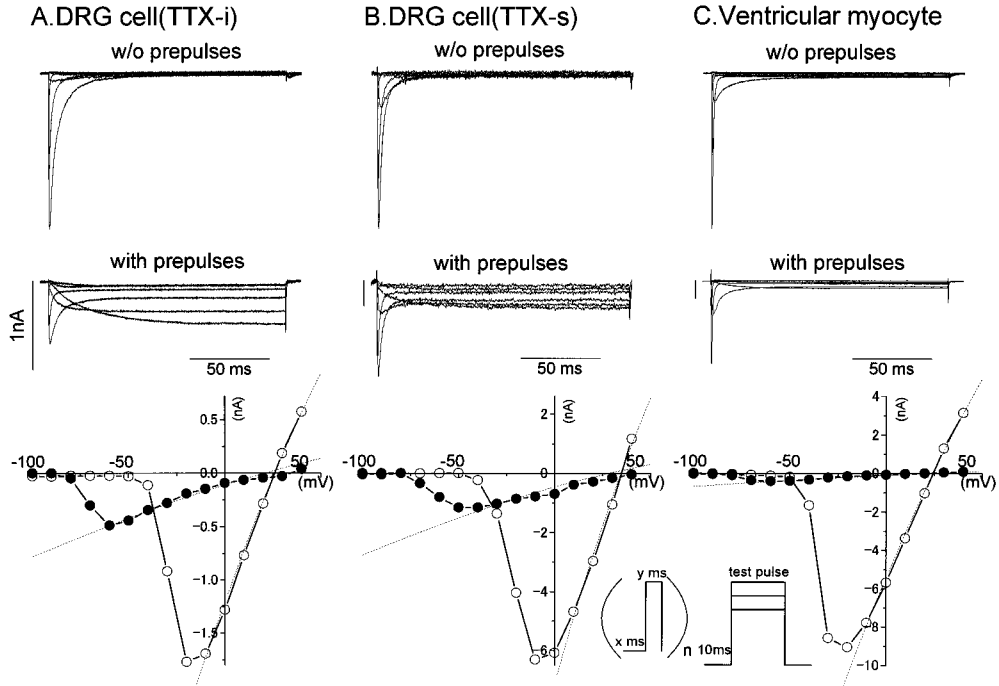


Fig. 3. Effect of 300 μM α-dihydro-GTX-II on macroscopic Na^+ currents recorded from DRG cells and ventricular myocytes. A–C, family of Na^+ currents associated with step depolarizations from −80 to +20 mV with a 20 mV step from a membrane potential of −140 mV is depicted under control conditions (without prepulses; upper row) and after GTX-evoked modification (with repetitive conditioning pulses; middle row). Current-voltage relationships are shown for control peak Na^+ currents (○) and for steady-state (modified) currents evoked by conditioning repetitive pulses in GTX (●)(bottom row). The dashed lines drawn were calculated by the GHK equation (see under *Dose-Response Relationship for GTX-Evoked Modification*). A, currents through TTX-i Na^+ channels from DRG cells. B, currents through TTX-s Na^+ channels in DRG cells. C, currents through Na^+ channels of ventricular myocytes. Pulse protocol for GTX-evoked channel modification is shown as inset (C; bottom row); the conditioning parameters, *x*, *y*, and *n*, are 8, 6, and 150 for A; 8, 3, and 300 for B; and 13, 5, and 1000 for C. Repetitive conditioning pulses for GTX-evoked channel modification always preceded each test pulse. Step test pulses (10 mV) of 160-ms duration were applied after holding the membranes at −140 mV for 10 ms. Interval between each combination of conditioning and test pulses ranged from 5 to 120 s.

without conditioning pulses. The maximum permeability for both control and GTX-modified Na^+ channels was computed by fitting the GHK equation to the current-voltage relationship at a range from +10 mV to +30 mV for control, and from −20 mV to +10 mV for GTX-modified Na^+ channels. In GHK equation:

$$I = A \cdot E \cdot \frac{[Na^+]_i - [Na^+]_o \cdot \exp\left(-E \cdot \frac{F}{RT}\right)}{1 - \exp\left(-E \cdot \frac{F}{RT}\right)}$$

I is the current amplitude, A is the permeability constant, E is the voltage, F is Faraday's constant, R is the gas constant, T is absolute temperature, and $[Na^+]_i$ and $[Na^+]_o$ are internal and external Na^+ concentrations, respectively. A is the only variable term, and the other terms remain constant regardless of the states of Na^+ channel. Thus, the normalized permeability constant—the ratio of permeability of control versus that of modified Na^+ channel—is equal to the normalized slope conductance. The dose-response curve for GTX-evoked modification of the Na^+ channels was constructed by plotting the normalized slope conductance against GTX concentration. As shown in Fig. 4, TTX-i Na^+ channels in DRG cells were the most sensitive to both GTX-I and α-dihydro-GTX-II. At the highest concentration employed (300 μM), the conductance of GTX-modified Na^+ channels reached as much as 21% (GTX-I) and 30% (α-dihydro-GTX-II) of that in control channels. The data were fitted with a sigmoidal curve, assuming 1:1 drug-receptor stoichiometry, as given by the equation:

$$\text{normalized slope conductance} = Y_{max}/(1 + EC_{50}/[GTX]_i),$$

where Y_{max} indicates the relative maximal permeability constant for GTX-modified Na^+ channels, EC_{50} denotes the half-maximal concentration, and $[GTX]_i$ represents the intracellular GTX concentration. The EC_{50} was estimated to be 31 μM for GTX-I and 54 μM for α-dihydro-GTX-II, respectively.

TTX-s Na^+ channels in DRG cells were far less sensitive to both GTX-I and α-dihydro-GTX-II than were TTX-i channels. Even at the highest concentration tested (300 μM), the normalized slope conductance reached only 43% (GTX-I) or 18% (α-dihydro-GTX-II) of the corresponding value for TTX-i Na^+ channels at the same toxin concentration. In addition, both GTX-I and α-dihydro-GTX-II were far less effective in modifying cardiac Na^+ channels than TTX-i Na^+ channels in DRG cells. In cardiomyocytes, the normalized slope conductance for the modified current in the presence of α-dihydro-GTX-II (300 μM) was 0.03 [i.e., about half of the corresponding value (0.08) in GTX-I at the same concentration]. It seems reasonable to conclude that the pharmacological action of α-dihydro-GTX-II in modifying DRG TTX-s channels and ventricular Na^+ channels was much weaker than that of GTX-I.

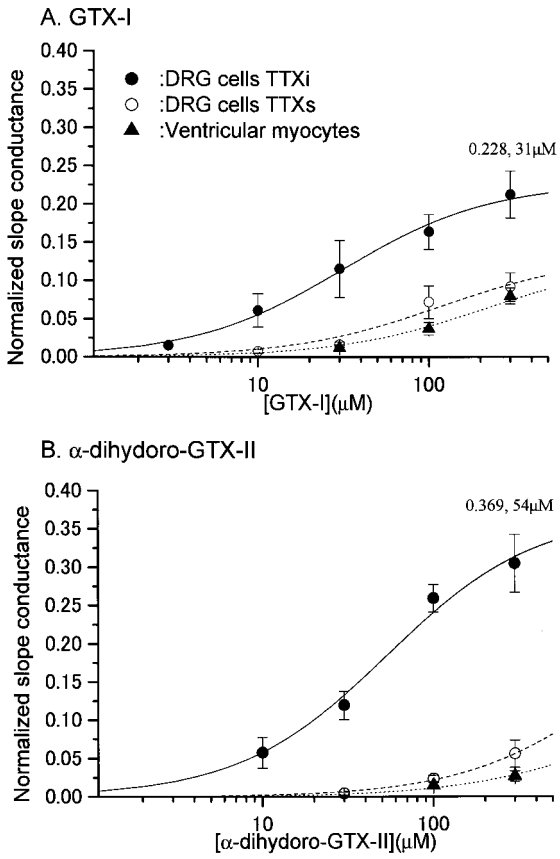


Fig. 4. Dose-response curves plotting normalized slope conductance of GTX-modified Na^+ channels against the concentration of GTXs. Solid line for DRG TTX-i Na^+ channels are curves of best fit to experimental data with EC_{50} = 31 μM (A), and EC_{50} = 54 μM for GTX-I α dihydro-GTX-II (B), respectively. Calculated maximal normalized slope conductance of TTX-i is 0.23 for GTX-I and 0.37 for α-dihydro-GTX-II, respectively. The dashed lines were drawn by eye. Data, in most instances, were taken from three to six cells.

Single-Channel Recordings

Reduced Single-Channel Conductance of GTX-Modified Na^+ Channels. Because whole-cell recordings showed that GTX analogs are much more potent in modifying TTX-i Na^+ channels from DRG cells than Na^+ channels from cardiomyocytes (see Fig. 3), we measured currents through single ion channels in an effort to compare the properties (single-channel conductance, probability of channel opening) of the modified Na^+ channels in DRG cells (TTX-i channels) and cardiomyocytes.

Before the formation of cell-attached patches, ventricular myocytes were preincubated in an external solution containing 100 μM GTX for 20 min, because GTX has been shown to have access to its binding site only after passing through the cell membrane (Seyama et al., 1988). Because the open time for GTX-modified ion channels is on the order of several tens of milliseconds and that for unmodified channels lasts as long as several milliseconds in selected records, we were able to obtain the single-channel current-voltage relationship directly by applying a ramp clamp. The ramp clamps used had a rising and falling slope of 8 V/s (voltage range, −120 to +40 mV) and were applied at a frequency of 1 Hz. Twenty consecutive recordings are displayed in Fig. 5A. During the first 10 episodes, single-channel currents were observed from time to time during the rising phase of the voltage ramp, but not during the falling phase, because of the full inactivation of the Na^+ channels (Hodgkin and Huxley, 1952), which were not yet modified. Evidence of channel modification can be seen at the eleventh episode: a single channel opening was followed by a sustained inward current, and this slow sustained current continued during the falling phase of the ramp. Successive ramp depolarizations (episodes 11 to 19)

produced a gradual change in inward current corresponding to the macroscopic sustained inward current observed under a whole-cell voltage clamp after a series of conditioning prepulses (Fig. 3). In analyzing the data obtained, records of inactivating single channel currents were taken to represent the current flowing through unmodified Na^+ channels, giving a value of 34 pS for single-channel conductance. In separate experiments, the conventional step pulse method for single-channel conductance also gave a value of 32 pS (data not shown). The noninactivating currents of prolonged duration were analyzed separately and represented currents through GTX-modified channels. The resultant current-voltage curves for the slow sustained current were concave in shape (Fig. 5C). A straight line was fitted to the data between −80 mV and −20 mV, and the single-channel conductance was determined from the slope. In this example, the single channel conductance of ventricular Na^+ channels was decreased from 34 pS to 11 pS after α-dihydro-GTX-II modification; Duch et al. (1992) reported a similar decrease in single channel conductance. All data for single Na^+ channel conductance before and after GTX modification are summarized in Table 1.

Effect of GTX Analogs on the P_o. Treatment with 100 μM α-dihydro-GTX-II induced a characteristic, long-lasting opening of single Na^+ channels (Fig. 6, A and D). Another noticeable finding is the presence of a clear subconductance state in Fig. 6A. This topic will be discussed in detail in a subsequent communication.

As shown in Fig. 6, B and E, the distribution of open times could be fitted by a single exponential function with a time constant, τ_o, of 286 ms (neuronal TTX-i channel) and 218 ms (ventricular Na^+ channels). Comparison of open time histograms constructed for Na^+ channels in DRG cells and in ventricular myocytes revealed no qualitative difference (Table 2). The closed time histogram for TTX-i Na^+ channels in DRG cells is best described as the sum of two exponential components, indicating that there are two closed states (Fig. 6C). By contrast, the closed time histogram for Na^+ channels in ventricular myocytes was well fitted by a single exponential function (Fig. 6F). Mean dwell times for GTX-modified Na^+ channels are summarized in Table 2. Because the channel openings did not overlap one another, $P_{o,GTX}$ was determined by dividing the total time spent in the open state by the pulse length. $P_{o,control}$ values for TTX-i Na^+ channels in

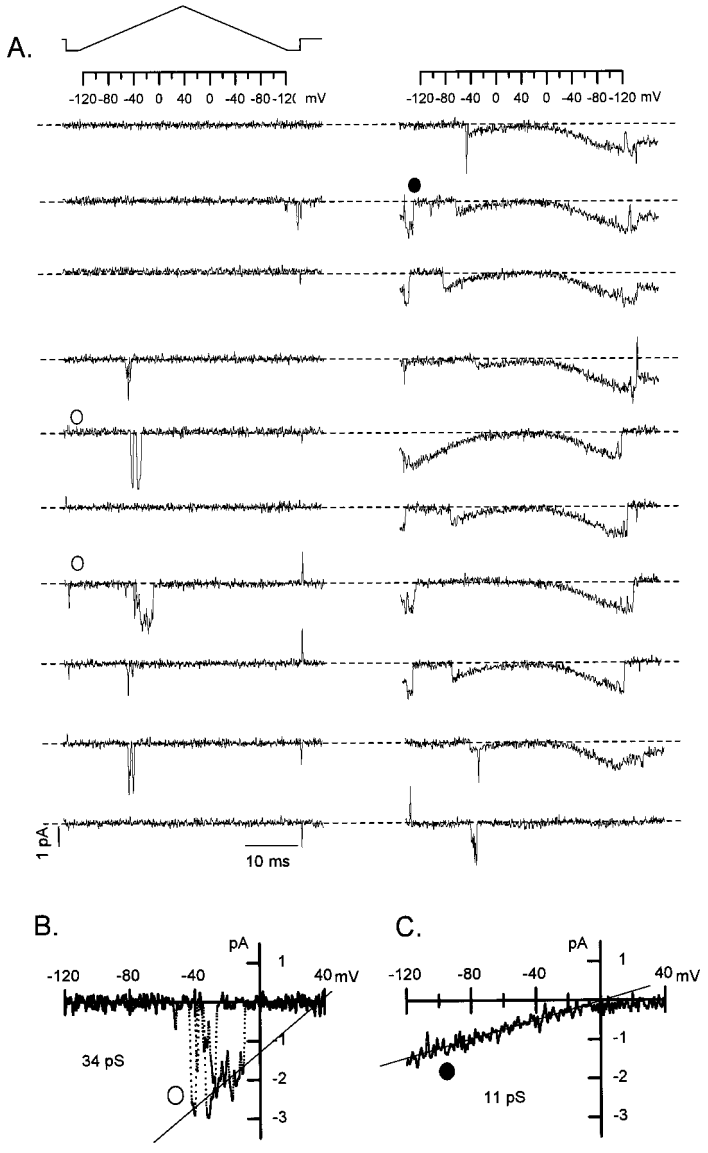


Fig. 5. Determination of single-channel conductance for control and modified Na^+ channels in a ventricular myocyte. A, sequential current records for a patch-clamped Na^+ channel in response to a voltage ramp, generated according to the protocol illustrated (inset); currents through the unmodified channels are shown in left column and those through the modified Na^+ channels in the right column. In B, two records in which a single unmodified channel opened during rising phase of voltage ramp (○) were superimposed; a straight line was fitted to the data. In C, a record of the modified current, obtained during the falling phase of ramp pulse (●), is displayed graphically along with the line of best fit. Slope of fitted lines directly gives the single channel conductance.

DRG cells (0.40) and those for the ventricular Na^+ channel (0.32; see under *Materials and Methods*), were in the same range. $P_{o,GTX}$ for ventricular and DRG TTX-i Na^+ channels, modified by both GTX analogs, were similar to each other regardless of the GTX analog tested (Table 2). The resemblance in $P_{o,GTX}$ for these Na^+ channels is ascribed to similar single channel kinetics (τ_o in B and C, and τ_c in C and F are close to each other in Fig. 6). Thus, it is thought that changes in P_o cannot explain the marked difference in GTX potency recorded in the whole-cell configuration that we observed.

Discussion

The present study has revealed the characteristics of GTX-evoked modification of Na^+ channels and differences in the efficiency of GTX-evoked modification among three Na^+ channel subtypes. First, GTX modifies the Na^+ channels only when repetitive conditioning depolarizing pulses precedes a test pulse. Second, the Na^+ channels modified by GTX can generate a slow sustained current that begins to flow at a membrane potential of −90 mV. Third, in the whole-cell

TABLE 1
Single Na^+ channels conductance

	$g_{control}$	g_{GTX}	$g_{GTX}/g_{control}$	Number of Experiments
	pS	*pS*		
Ventricular Na^+ channel				
GTX-I	31.1 ± 2.7	7.8 ± 0.8	0.25 ± 0.04	11
α-Dihydro-GTX-II	31.7 ± 2.4	10.7 ± 1.0	0.34 ± 0.05	5
DRG TTX-i Na^+ channel				
GTX-I	25.9 ± 4.4	7.8 ± 1.1	0.31 ± 0.06	5
α-Dihydro-GTX-II	28.0 ± 5.6	11.6 ± 2.1	0.42 ± 0.03	4

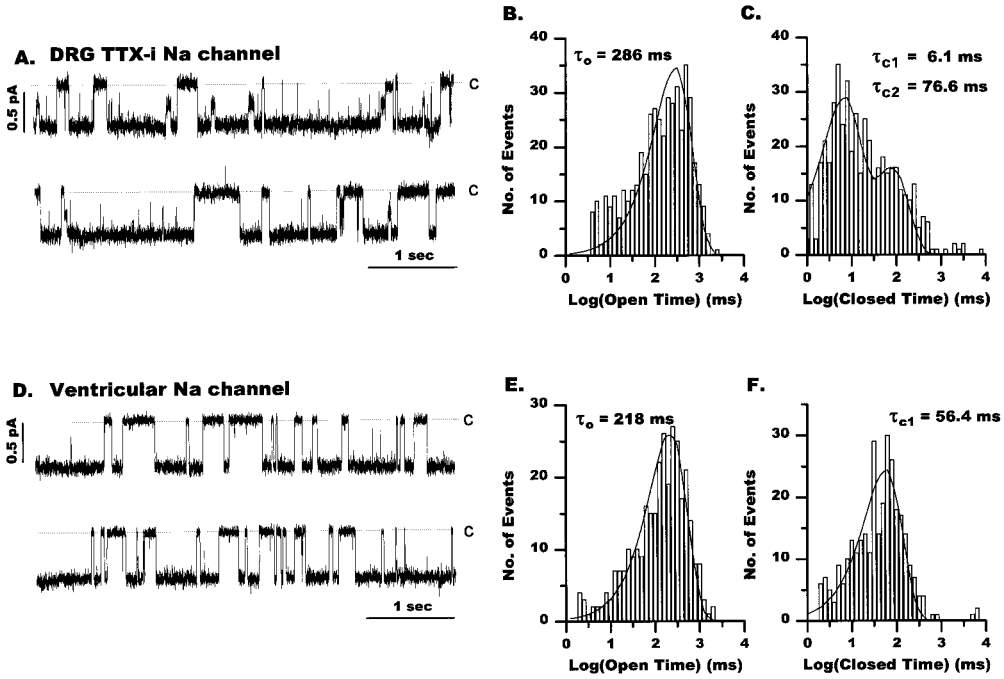


Fig. 6. Analysis of single-channel currents modified by α-dihydro-GTX-II obtained in neuronal (DRG) TTX-i Na^+ channel (A, B, and C) or ventricular Na^+ channel (D, E, and F). A and D, representative single-channel current records from DRG TTX-i (A) and ventricular (D) cells. Symbol c (in A and D) marks the closed channel (zero current) level. B and E, open time histograms from single-channel recordings, including segments shown in A and D, were fitted by a single exponential function with indicated time constants. C and F, closed time histograms from single-channel recordings, including segments shown in A and D, were fitted either by a single exponential or by the sum of two exponentials, as appropriate, with indicated time constants. Membrane potentials in each cell-attached experiment were −0.7 ± 1.4 mV (n = 3) for DRG cells and 2.7 ± 2.0 mV (n = 3) for ventricular cells using glass microelectrodes.

TABLE 2
Kinetic parameters for single Na^+ channels modified by GTXs

	τ_o	τ_{c1}	τ_{c2}	$P_{o,GTX}$	Number of Experiments
	ms	*ms*	*ms*		
Ventricular Na^+ channel					
GTX-I	53.9 ± 13.3	13.3 ± 6.7	196.1 ± 102.3	0.50 ± 0.07	9
α-Dihydro-GTX-II	178.2 ± 92.9	65.1 ± 20.8		0.70 ± 0.11	6
DRG TTX-i Na^+ channel					
GTX-I	104.7 ± 42.9	13.7 ± 3.9	106.0 ± 70.2	0.65 ± 0.11	4
α-Dihydro-GTX-II	212.9 ± 67.9	6.3 ± 1.7	66.3 ± 24.0	0.71 ± 0.02	4

variation of the patch clamp technique, the degree of GTX-evoked modification is measured as the relative maximum permeability for modified Na^+ channels referred to that for Na^+ channels without conditioning pulses. TTX-i Na^+ channels (DRG cells) were the most sensitive to both GTX analogs. Other TTX-s and ventricular Na^+ channels proved to be far less sensitive to GTX analogs. The most striking difference in the response of Na^+ channels to GTX analogs was observed between the neuronal TTX-i and ventricular Na^+ channels, using α-dihydro-GTX-II to modify the channels. Fourth, to investigate the factors responsible for the observed difference in the response of DRG cells and cardiomyocytes to α-dihydro-GTX-II and GTX-I, the induced changes in single channel conductance and P_o was examined by the patch-clamp method. As summarized in Tables 1 and 2, there is virtually no difference in either parameter, after preparations were subjected to GTX-I or α-dihydro-GTX-II.

Generally, the whole-cell conductance (G) is expressed as $N_{total} \times P_o \times g$, where N_{total} is the number of functioning Na^+ channels in a cell, and g is the single-channel conductance. For the reader's convenience, the additional subscript attached to these symbols represents the experimental condition. The maximum conductance at the time of peak Na^+ current in control ($G_{control}$) is expressed as $N_{total} \times P_{o,control} \times g_{control}$, and the whole-cell conductance of the Na^+ channels under conditions in which all the channels are modified by GTX (G_{GTX}) is $N_{total} \times P_{o,GTX} \times g_{GTX}$. Thus, the relative maximum conductance, $G_{GTX}/G_{control}$, is calculated as $(P_{o,GTX} \times g_{GTX})/(P_{o,control} \times g_{control})$, where the values for N_{total} in the numerator and denominator were cancelled out. Because there is a large discrepancy between the calculated and the observed values of maximum normalized slope conductance (in Table 3) and all the parameters used to calculate the theoretical values were, with the exception of the N_{total} parameter, determined experimentally, it is reasonable to suppose that our starting assumption that all the Na^+ channels were modified by GTX is no longer valid. Thus, a major factor responsible for the wide variation in the response to the two GTX analogs in the whole-cell recording is suggested to be the number of Na^+ channels modified by GTX analogs.

From these calculations, the modified fraction of Na^+ channels in neuronal TTX-i and ventricular Na^+ channels has been estimated to be 42% and 21%, respectively, in 300 μM GTX-I, and 40% and 4%, respectively, in 300 μM α-dihydro-GTX-II. There is a large difference in potency among the GTX analogs used. For example, α-dihydro-GTX-II modified only 4% of Na^+ channels in ventricular myocytes, whereas GTX-I modified 42% of TTX-i Na^+ channels in DRG cells. The most likely explanation is that, once bound, these two biologically active GTX analogs had a similar capacity to modify Na^+ channels, but the ease with which GTX analogs can bind and unbind at the site of action could differ. In support of this explanation, α-dihydro-GTX-II molecules unbind three times faster than GTX-I molecules from ventricular Na^+ channels. One could speculate that the marked difference in dissociation rates between GTX-I and α-dihydro-GTX-II is caused by the different molecular species occupying the C-14R position (Tsuji et al., 1991): GTX-I presents an acetyl group and α-dihydro-GTX-II presents a hydroxyl group. The more hydrophilic hydroxyl group (or its smaller molecular radius) could promote more rapid dissociation of α-dihydro-GTX-II from its binding site.

It has been shown that the primary structure of rat DRG TTX-i Na^+ channels is akin to that of rat cardiac Na^+ channels (Akopian et al., 1996), and the response to biological toxins, such as TTX, venoms from Australian funnel-web spiders (Nicholson et al., 1994), and μ-conotoxin GIIIA is much stronger in TTX-s Na^+ channels than that in TTX-i Na^+ channels or cardiac Na^+ channels (Moczydlowski et al., 1986). On the contrary, both GTX analogs give rise to more pharmacologically conspicuous response in DRG TTX-i Na^+ channels compared with those in DRG TTX-s and cardiac Na^+ channels. Because Yamaoka (1987) showed that frog ventricular Na^+ channels are suppressed by TTX with an IC_{50} value of 27 nM, which is 2 orders of magnitude lower than the IC_{50} value of several micromolar for the mammalian ventricular Na^+ channels, frog ventricular Na^+ channels are pharmacologically close to the group of TTX-s Na^+ channels.

TABLE 3

Comparison of normalized slope conductance calculated by data from single-channel measurement and derived from whole-cell experiments

The numbers in parenthesis are experimented cells.

	$\frac{P_{o,GTX} \times g_{GTX}}{P_{o,control} \times g_{control}}$	Normalized Slope Conductance
Ventricular Na^+ channel		
GTX-I	0.39	0.08 ± 0.01 (6)
α-dihydro-GTX-II	0.74	0.03 ± 0.01 (6)
DRG TTX-i Na^+ channel		
GTX-I	0.50	0.21 ± 0.03 (4)
α-dihydro-GTX-II	0.75	0.30 ± 0.04 (3)

Acknowledgments

We thank Dr. Stephen M. Vogel for critical reading of the manuscript.

References

Akopian AN, Sivilotti L and Wood JN (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature (Lond)* **379:**257–262.
Aldrich RW, Corey DP and Stevens CF (1983) A reinterpretation of mammalian sodium channel gating based on single channel recording. *Nature (Lond)* **306:**436–441.
Campbell DT (1992) Large and small vertebrate sensory neurons express different Na and K channel subtypes. *Proc Natl Acad Sci USA* **89:**9569–9573.
Catterall WA (1980) Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharmacol Toxicol* **20:**15–43.
Duch DS, Hernandez A, Levinson SR and Urban BW (1992) Grayanotoxin-I-modified eel electroplax sodium channels. Correlation with batrachotoxin and veratridine modifications. *J Gen Physiol* **100:**623–645.
Goldman DE (1943) Potential, impedance, and rectification in membranes. *J Gen Physiol* **27:**37–60.
Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pfluegers Arch* **391:**85–100.
Hille B (1968) Pharmacological modifications of the sodium channels of frog nerve. *J Gen Physiol* **51:**199–219.
Hodgkin AL and Huxley AF (1952) A quantitative description of membrane current and its application to conductance in nerve. *J Physiol Lond* **117:**500–544.
Hodgkin AL and Katz B (1949) The effect of sodium ions on the electrical activity of the giant axon of the squid. *J Physiol (Lond)* **108:**37–77.
Khodorov BI (1985) Batrachotoxin as a tool to study voltage-sensitive sodium channels of excitable membranes. *Prog Biophys Mol Biol* **45:**57–148.
Moczydlowski E, Olivera BM, Gray WR and Strichartz GR (1986) Discrimination of muscle and neuronal N-channel subtypes by binding competition between [^{3}H]saxitoxin and μ-conotoxins. *Proc Natl Acad Sci USA* **83:**5321–5325.
Narahashi T and Herman MD (1992) Overview of toxins and drugs as tools to study excitable membrane ion channels: I. Voltage-activated channels. *Methods Enzymol* **207:**620–643.
Narahashi T, Roy ML and Ginsburg KS (1994) Recent advances in the study of mechanism of action of marine neurotoxins. *Neurotoxicology* **15:**545–554.
Nicholson GM, Walsh R, Little MJ and Tyler MI (1998) Characterization of the effects of robustoxin, the lethal neurotoxin from the Sydney funnel-web spider *Atrax robustus*, on sodium channel activation and inactivation. *Pfluegers Arch* **436:**117–126.
Nicholson GM, Willow M, Howden ME and Narahashi T (1994) Modification of

sodium channel gating and kinetics by versutoxin from the Australian funnel-web spider *Hadronyche versuta*. *Pfluegers Arch* **428:**400–409.
Noda M, Shimizu S, Tanabe T, Takai T, Kayano T, Ikeda T, Takahashi H, Nakayama H, Kanaoka Y, Minamino N, Kangawa K, Matsuo H, Raftery MA, Hirose T, Inayama S, Hayashida H, Miyata T and Numa S (1984) Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. *Nature (Lond)* **312:**121–127.
Scholz A, Appel N and Vogel W (1998a) Two types of TTX-resistant and one TTX-sensitive Na^+ channel in rat dorsal root ganglion neurons and their blockade by halothane. *Eur J Neurosci* **10:**2547–56.
Scholz A, Kuboyama N, Hempelmann G and Vogel W (1998b) Complex blockade of TTX-resistant Na^+ currents by lidocaine and bupivacaine reduce firing frequency in DRG neurons. *J Neurophysiol* **79:**1746–54.
Seyama I, Yamada K, Kato R, Masutani T and Hamada M (1988) Grayanotoxin opens Na channels from inside the squid axonal membrane. *Biophys J* **53:**271–274.
Seyama I and Yamaoka K (1988) A study of the electrical characteristics of sodium currents in single ventricular cells of the frog. *J Physiol (Lond)* **401:**257–275.
Tsuji K, Kawanishi T, Handa S, Kamano H, Iwasa J and Seyama I (1991) Effect of structural modification of several groups on the D-ring of grayanotoxin on its depolarization potency in squid giant axon. *J Pharmacol Exp Ther* **257:**788–794.
Wu JV and Kendig JJ (1998) Differential sensitivities of TTX-resistant and TTX-sensitive sodium channels to anesthetic concentrations of ethanol in rat sensory neurons. *J Neurosci Res* **54:**433–443.
Yakehiro M, Seyama I and Narahashi T (1997) Kinetics of grayanotoxin-evoked modification of sodium channels in squid giant axons. *Pfluegers Arch* **433:**403–412.
Yamaoka K (1987) Does the maximum upstroke velocity of the action potential (Vmax) represent available sodium conductance in frog ventricular cells? *Jpn J Physiol* **37:**585–599

Send reprint requests to: Dr. Issei Seyama, Department of Physiology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Hiroshima 734-8551, Japan. E-mail: issei@mcai.med.hiroshima-u.ac.jp