Brevetoxin-3 (PbTx-3) and Its Derivatives Modulate Single Tetrodotoxin-Sensitive Sodium Channels in Rat Sensory Neurons

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ABSTRACT

Brevetoxin-3 (PbTx-3), produced by marine dinoflagellates (Pychodiscus brevis), is a lipophilic 11-ring polyether molecule that binds with high affinity to site 5 of the voltage-sensitive sodium (Na+) channel. The effects of PbTx-3 and its derivatives were studied in cell-attached membrane patches on neurons dissociated from neonatal rat nodose ganglia by the patch-clamp technique. PbTx-3 (30–500 nM) produced a shift in conductances of 10.7 pS and 21.2 pS. PbTx-3 inhibits the inactivation of Na+ channels and prolongs the mean open time of these channels. Unitary Na+ currents could be blocked by μM tetrodotoxin (TTX) added to the pipette solution, which indicates that the single-channel currents are caused by the opening of TTX-sensitive Na+ channels. The PbTx-3 molecule is proposed to have multiple active centers (A-ring lactone, C-42 of R side chain) interacting with the Na+ channel binding site. Modification of the molecular structure of PbTx-3 at these centers produced derivatives (PbTx-6, 2,3,41,43-tetrahydro-PbTx-3, 2,3,27,28,41,43-hexahydro-PbTx-3 and 2,3-dihydro-PbTx-3 A-ring diol), which were less potent than PbTx-3 in producing similar effects on Na+ channel kinetics. PbTx-3 and its derivatives may provide insight into the mechanics of voltage-sensitive Na+ channel gating.

The brevetoxins are multi-ring polyether neurotoxins produced by marine dinoflagellates (Pychodiscus brevis = Gymnodinium breve) found in Florida waters (Baden, 1988) and more recently in New Zealand waters (Ishida et al., 1995). A human disease known as neurotoxic shellfish poisoning has been ascribed to the consumption of shellfish contaminated with these toxins (Baden, 1988).

Brevetoxins possess a molecular structure consisting of 11 transfused rings, 23 stereocenters and an overall linear low-energy conformation. The total synthesis of brevetoxin B in its naturally occurring form has been reported recently, as have shorter molecules that contain all of the salient features of the natural toxin, except they are shorter in length (Nicolau et al., 1994, 1995). The brevetoxins have been used as model ligands for a more serious neurointoxication phenomenon known as ciguatera. Ciguatera is a human disease associated with the consumption of reef fishes that have accumulated the structurally related marine polyether toxin known as ciguatoxins (Lewis and Holmes, 1993).

Both the brevetoxins and ciguatoxins exert their neurotoxicity by altering the gating and sodium ion permeability of voltage-sensitive Na+ channels in excitable membranes (Baden, 1989; Wu and Narahashi, 1988). Numerous studies have investigated the effects of purified toxins on ion channel binding and electrophysiology, and it is known that both toxin groups bind specifically to site 5 associated with domain IV of the voltage-gated sodium channel alpha subunit (Poli et al., 1986; Rein et al., 1994; Trainer et al., 1991, 1994). It is believed, based on various lines of evidence, that these polyether toxins orientate across the plasma membrane, parallel to selected helices in the alpha subunit of the channel (Gawley et al., 1995; Trainer et al., 1994). Orientation of the

ABBREVIATIONS: PbTx-3, brevetoxin from Pychodiscus brevis; TTX, tetrodotoxin; TEA, tetraethylammonium ions; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; DIDS, 1,4,4’-diiodocyanato-2,2’-stilbenedisulfonic acid disodium salt.
brevetoxins is said to be “head-down” with the A-ring lactone facing the cell interior and the “tail” end of the molecule facing outward. These hypothetical features are supported by photoaffinity probe covalent modification (Trainer et al., 1991, 1994) and preliminary electrophysiological studies (Gawley et al., 1995).

As part of our continuing efforts to describe the effects of the brevetoxins on voltage-sensitive Na$^{+}$ channels at the molecular level, the effects of natural toxins on single Na$^{+}$ channels were examined under patch-clamp conditions. Appropriate brevetoxin derivatives were then specifically designed and synthesized in our laboratories, based on results of computer molecular modeling and specific radioreceptor protocols, and examined electrophysiologically. A preliminary report of some of these results has been presented (Jeglitsch et al., 1994).

**Materials and Methods**

**Preparation.** Sensory neurons from rat nodose ganglia were isolated as described previously (Baden et al., 1994). The nodose ganglia were dissected from neonatal rats (1–2 weeks old), sacrificed by intraperitoneal injection of sodium pentobarbital and incubated in a Krebs' solution containing collagenase (0.6 mg ml$^{-1}$, type 2; Worthington Biochemical Corp., Freehold, NJ) and protease (0.4 mg ml$^{-1}$, Sigma Chemical Co., St. Louis, MO) for 1 hr at 37°C. The ganglia were transferred to a sterile culture medium (Dulbecco's Modified Eagle's Medium, 10% (v/v) fetal calf serum, 100 U ml$^{-1}$ penicillin, 0.1 mg ml$^{-1}$ streptomycin) and mechanically dissociated by a pair of forceps. The tissue fragments were triturated with a sterile fine-bore Pasteur pipette, and the dissociated neurons were plated onto laminin-coated 18-mm glass coverslips. The dissociated cells were incubated at 37°C in a 5% CO$_2$ atmosphere, and electrophysiological recordings were made from neurons that had been maintained in tissue culture 36 to 72 hr. Isolated nodose ganglion neurons studied had diameters of 15 to 25 μm.

**Electrophysiological recording.** Single Na$^{+}$ channel currents were recorded from cell-attached membrane patches by the patch-clamp technique (Hamill et al., 1981). Patch pipettes were fabricated from thick-walled borosilicate glass (GC150F, Clark Electromedical Instruments, Reading, UK) and the tip was fire-polished and coated with Silgard (Dow Corning, Midland, MI) to reduce electrode capacitance. Pipettes had tip resistances of 10 to 15 megohm when filled with patch solution. Patch electrodes sealed against the cell membrane typically had seal resistances of 5 to 10 gigaohm. Voltage steps were applied with pulse protocols generated by a PC computer (Dell 486/50 MHz) and pClamp programs (Axon Instruments Inc., Foster City, CA). The transmembrane potential was initially held at −100 mV (the measured resting membrane potential was −50 mV) before depolarizing voltage steps were applied. Single-channel currents were recorded with an Axopatch 200A patch-clamp amplifier (Axon Instruments Inc., Foster City, CA), filtered at 2 kHz (−3 dB, 4-pole low-pass Bessel filter) and sampled at 10 kHz with an A/D converter (TL-1 Labmaster DMA interface) and computer with pClamp programs. Linear leak and capacitative currents were subtracted from current traces with records that contained no channel openings during the depolarizing pulse. Steady-state single-channel currents were recorded on videotape with an A/D recorder adaptor (PCM-2, Medical Systems, Greenvile, NY) before computer analysis.

The resting membrane potential and depolarization-activated Na$^{+}$ currents were monitored with the whole-cell recording configuration. Whole-cell recordings were achieved by rupturing the membrane patch with patch pipettes (2–6 megohm) filled with an appropriate internal solution as described below. The resting membrane potential was measured within 5 min upon obtaining the whole-cell recording configuration. The isolated neurons were voltage-clamped at −90 mV before depolarizing command pulses (60 ms duration) were applied. Whole-cell membrane currents were filtered with a 4-pole Bessel filter at 5 kHz (−3 dB) and sampled at 33 kHz.

Unitary currents records were transferred from video tape to a computer with a threshold-detection device (AI202A event detector) and pClamp acquisition (version 5.5.1; Axon Instruments Inc.) and Filecat programs. Analysis of unitary currents was performed by pClamp programs, Fetchan and pSTAT. Unitary current amplitude distributions were obtained by measuring the difference between the base-line current and amplitude of each event which contributed one point to the amplitude distribution for the patch. Single Na$^{+}$ channel kinetics (apparent mean open times and open probability) were determined from the idealized unitary current records by threshold detection for channel openings set at 50% of the amplitude of a single-channel opening, and a minimum resolvable time of 200 μs. Normalized NP$^{open}$ (product of number of channels and single-channel open probability) was determined from the maximum values obtained in the absence (control) and presence of 100 nM PbTx-3. Numerical data are expressed as the mean ± S.E. (n, number of observations).

**Solutions and toxins.** The composition of the physiological salt solution was: 140 mM NaCl, 3 mM KCl, 0.6 mM MgCl$_2$, 2.5 mM CaCl$_2$, 7.7 mM glucose, 10 mM histidine, adjusted to pH 7.4 with NaOH. The extracellular solution used for whole-cell recordings contained: 50 mM NaCl, 3 mM MgCl$_2$, 0.1 mM CdCl$_2$, 3 mM KCl, 90 mM TEA-Cl, 7.7 mM glucose, 10 mM HEPES adjusted to pH 7.4 with TEA-Cl. A series of experiments, whole-cell recordings were made in the presence of 1 μM TTX added to the bath solution. The intracellular pipette solution for whole-cell recordings contained: 140 mM NaCl, 90 mM CsCl, 2 mM Mg$_2$ATP, 10 mM EGTA, 7.7 mM glucose, 10 mM HEPES adjusted to pH 7.2 with CsOH. The internal pipette solution contained a high Cs$^+$ concentration and the external bath solution contained TEA to block K$^+$ channels. To suppress depolarization-activated Ca$^{2+}$ currents, EGTA and F$^{-}$ were used to buffer intracellular Ca$^{2+}$, and extracellular Ca$^{2+}$ was replaced by Mg$^{2+}$. Ca$^{2+}$ currents were further suppressed by the addition of 100 μM Cd$^{2+}$ to the external solution. Cd$^{2+}$ was used at low concentrations (100 μM) because Cd$^{2+}$ has been reported to reduce the TTX-resistant Na$^{+}$ current by 50% at concentrations >500 μM (Ikedo et al., 1986).

The extracellular solution used for single-channel recordings from cell-attached membrane patches contained: 140 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$, 0.6 mM MgCl$_2$, 7.7 mM glucose and 10 mM HEPES adjusted to pH 7.4 with NaOH. The patch pipette solution used for cell-attached single-channel recordings contained: 140 mM NaCl, 3 mM KCl, 0.6 mM MgCl$_2$, 2.5 mM BaCl$_2$, 30 mM TEA-Cl, 7.7 mM glucose, 0.1 mM DIDS and 10 mM HEPES adjusted to pH 7.4 with NaOH. The osmolality of the extracellular and patch pipette solutions (285–290 mosmol kg$^{-1}$) was monitored with a vapor pressure osmometer (Wescor 5500, Logan, UT). For single-channel recordings obtained in the presence of toxins, TTX, brevetoxin PbTx-3 or one of the PtX-derivatives (PbTx-6, compounds 1–3) were added to the pipette solution at the concentrations stated. TTX (Calbiochem, San Diego, CA) was dissolved in distilled H$_2$O, and PbTx-3 and derivatives were dissolved in absolute ethanol. Experiments were conducted at room temperature (22–23°C).

All toxin derivatives were obtained by chemical modification of the molecular structure of native PbTx-3 (C$_{56}$H$_{72}$O$_{44}$). Natural toxins were obtained from laboratory cultures of the toxigenic organism, strain WB 58. The following derivatives were studied and their structures are shown in figure 1: PbTx-6, 2,3,4,11-tetrahydro-PbTx-3 (compound 1), 2,3,27,28,41,43-hexahydro-PbTx-3 (compound 2) and 2,3-dihydro-PbTx-3 A-ring diol (compound 3). The identity of each toxin derivative was ascertained with 400 MHz $^1$H and $^{13}$C nuclear magnetic resonance spectrometry. All derivatives were tested in a rat brain synaptosome binding system (Poli et al., 1986) to be certain there was high-affinity specific binding to site 5, and all
Preliminary voltage-clamp experiments were carried out on neonatal rat nodose ganglion cells to determine the TTX sensitivity of the whole-cell Na⁺ current. The average resting membrane potential measured in physiological salt solution was \(-51.1 \pm 1.0\) mV \((n = 15)\). In the absence of TTX, inward Na⁺ currents activate at potentials more depolarized than \(-50\) mV, reach a maximum amplitude at approximately \(-20\) mV and reversed near \(+40\) mV. In the presence of 1 μM TTX, no current was obtained upon step depolarization in 10 mV increments from \(-90\) mV to \(+50\) mV \((n = 15,\) data not shown). The effect of TTX was reversible, with the inward Na⁺ current reappearing upon washout of TTX.

Isolation of single Na⁺ channel currents in cell-attached membrane patches. To separate single Na⁺ channel currents from K⁺, Ca⁺⁺ and Cl⁻ currents, specific pharmacological agents were added exclusively to the pipette solution. K⁺ currents were inhibited by the addition of Ba⁺⁺ and TEA, and Cl⁻ channels were blocked by adding 100 μM DIDS to the pipette solution. Ca⁺⁺ currents were suppressed by substituting Mg²⁺ for Ca⁺⁺ in the bath solution.

The cell-attached recording configuration was used to study the unmodified and toxin-modified, voltage-dependent Na⁺ currents at the single-channel level. No single-channel activity was observed in any cell-attached membrane patch in response to depolarizing voltage steps, if the patch pipette was filled with 1 μM TTX \((n > 20)\). In some experiments, to investigate the toxin-modified Na⁺ channel, the pipette tip was filled with a solution containing PbTx-3 and the pipette was back-filled with the same solution containing 1 μM TTX, to determine the TTX sensitivity of the Na⁺ channel studied \((Roy et al., 1994)\). The diffusion of TTX to the membrane patch inhibited the Na⁺ channel activity observed in the presence of PbTx-3 in all membrane patches studied, which indicates that the Na⁺ channels studied were TTX sensitive.

**Results**

Acute PbTx-3-modified and unmodified Na⁺ channels. The molecular structures of brevetoxin B and derivatives that bind to “site 5” of the voltage-gated sodium channel. The structures of brevetoxin-3 (PbTx-3), PbTx-6 and the derivatization of PbTx-3 to produce the derivatives, 2,3-dihydro-PbTx-3 A-ring diol (compound 3), 2,3,27,28,41,43-hexahydro-PbTx-3 (compound 2) and 2,3,41,43-tetrahydro-PbTx-3 (compound 1) used in this study are shown.

Derivatives were assessed for whole-animal potency by *Gambusia* fish bioassay \((Rein et al., 1994)\).

**Activation and inactivation kinetics of PbTx-3-modified and unmodified Na⁺ channels.** In the absence of PbTx-3, no single-channel activity was observed in membrane patches under steady-state conditions; that is, during maintained depolarization (data not shown). Under control conditions, voltage-dependent Na⁺ channels can only be observed following step depolarizations applied to the membrane patch. Brief \((\leq 5\) ms), single-channel openings were observed immediately after a depolarizing step (fig. 2A). Maintained depolarization at voltages more positive than \(-30\) mV inactivated the Na⁺ channels and no channel openings were observed.

In the presence of 100 nM PbTx-3, increased single-channel activity was observed, and single-channel currents occurred throughout the depolarizing step. Long-duration channel openings \((>5\) ms) were also observed during maintained depolarization (fig. 2B). The Na⁺ channel appears to switch between the closed and open conformations but does not inactivate. PbTx-3, a lipid-soluble Na⁺ channel activator \((Baden 1989,\) Wu and Narahashi, 1988), appears to inhibit Na⁺ channel inactivation and increases the mean apparent channel open time. The effect of PbTx-3 on Na⁺ channel inactivation and apparent open time were irreversible.

Figure 2, C and D, shows the ensemble averages (below the corresponding traces) of records obtained under control conditions and in the presence of 100 nM PbTx-3. The ensemble average inward Na⁺ current obtained from the control records (fig. 2C) is comparatively small because of fewer single-channel events after a depolarizing pulse. The ensemble average current obtained from channel openings recorded in the presence of PbTx-3 (modified Na⁺ channel) (fig. 2D) has a larger amplitude because of an increased number of openings. The rate of decay of the macroscopic inward Na⁺ current was slowed, and a noninactivating component was apparent in the presence of PbTx-3. The noninactivating inward current shown in figure 2D (the base line is indicated by the dashed line) is caused by averaged late open events of the PbTx-3-modified Na⁺ channel.

The total apparent open times of the unmodified and PbTx-3-modified Na⁺ channel are compared in figure 3A. The normalized total apparent open times obtained at various membrane potentials are shown. The open probability is increased significantly in the presence of the channel modi-
fier, PbTx-3. PbTx-3 increased the total apparent open time and shifted the Na$^+$ channel activation curve to more negative membrane potentials. The normalized open probabilities (NP_open) of the modified and unmodified Na$^+$ channels are shown in figure 3B. In the presence of 100 nM PbTx-3, the activation curve is shifted by approximately −10 mV with respect to the activation curve obtained in the absence of PbTx-3 (control).

**Fig. 2.** Depolarization-activated single sodium channel currents in the absence (A) and presence (B) of 100 nM PbTx-3. (A, B) Typical consecutive traces (200 ms duration) of unitary Na$^+$ currents (downward deflections) obtained on depolarization to −30 mV from −100 mV (resting membrane potential = −50 mV) in the absence (Control) and presence of 100 nM PbTx-3. The voltage jump protocol is shown above the current traces. Pipette and bath solutions are as described under “Materials and Methods.” (C, D) Ensemble average of summarized Na$^+$ inward current obtained in the absence (Control; C) and presence of 100 nM PbTx-3 (D) by averaging 40 consecutive traces as shown in A and B, respectively. The dashed line indicates the zero-current level.

**Single Na$^+$ channel events observed under steady-state conditions.** PbTx-3 has been reported to inhibit the inactivation of voltage-sensitive Na$^+$ channels (Baden, 1989; Wu and Narahashi, 1988). The modified Na$^+$ channel can be studied not only with voltage jumps but also under steady-state conditions (see fig. 4A). Inhibition of Na$^+$ channel inactivation by PbTx-3 can be observed during maintained depolarization at −50 mV for several minutes (see fig. 5 in Baden et al., 1994). PbTx-3 stabilizes the open conformation whereby channel openings can be observed during the entire period. The modified channel switches between the closed and open conformations and does not appear to inactivate. Although the Na$^+$ channel activity remains unchanged during the course of the recording, the unitary currents were inhibited if the patch pipette was backfilled with 1 μM TTX (see above).

**Sublevel gating and slope conductances.** In the absence of PbTx-3, the brief Na$^+$ channel openings observed on step depolarization of cell-attached patches had a single-channel conductance of ~21 pS. PbTx-3, unlike other known gating modifiers of the voltage-dependent Na$^+$ channel, is able to stabilize more than one distinct conductance level (Schreibmayer and Jeglitsch, 1992). Figure 4A shows PbTx-3-induced sublevel behavior recorded under steady-state conditions at different membrane potentials. The slope conductances determined for the two conductance levels were 10.7 ± 0.8 pS for level I and 21.2 ± 0.6 pS for level II ($n = 3$, fig. 4B). The single-channel conductance (g_Na) for level II is twice that of level I. The extrapolated reversal potentials obtained for the unitary current-voltage relationships in the absence and presence of PbTx-3 were similar (~ +60 mV) and as predicted by the Nernst equation for a Na$^+$-selective electrode, which suggests that the ionic selectivity of the Na$^+$ channel is not modified by PbTx-3.

The amplitude histogram of single-channel events obtained from a continuous recording at the resting membrane
inactivation and shifts the activation curve but it also
total open time of the Na$^{+}$ absence (Control) and presence of 100 nM PbTx-3. (A) The normalized open probability of the voltage-dependent Na$^{+}$ channel calculated from the data shown in A. The Na$^{+}$ channel open probability (NPopen) is fit by a Boltzmann distribution with midpoints of $-20$ mV and $-30$ mV and slope parameters of 5.2 and 4.2, respectively.

Potential, $-50$ mV, shows three maxima: one at 0 pA (base line), a second at 1.16 pA (level I) and a third at 2.27 pA (level II) (fig. 4C). The amplitude histogram was best fit by three Gaussian functions (dotted lines). Transitions between the two open states were occasionally observed.

**Apparent open time of the unmodified and PbTx-3-modified Na$^{+}$ channel.** PbTx-3 not only inhibits Na$^{+}$ channel inactivation and shifts the activation curve but it also increases the open-channel probability. The increase in Na$^{+}$ channel open probability is caused by a decrease in the closed time between individual openings on depolarization and an increase in the single-channel open time as shown in figure 5A. Under control conditions, only brief openings are observed immediately after step depolarizations. In the presence of PbTx-3, Na$^{+}$ channel openings are observed during maintained depolarization and individual channel openings have significantly longer open times than those of the unmodified Na$^{+}$ channel. PbTx-3 appears to stabilize the open (conducting) conformation in a concentration-dependent manner. The highest channel activity was observed in the presence of 500 nM PbTx-3, whereas 3 nM PbTx-3 had no effect on Na$^{+}$ channel activity (data not shown). The mean apparent open time ($\tau_o$) was increased in the presence of both 30 and 500 nM PbTx-3 with a maximum increase in $\tau_o$ of $\sim$80% in the presence of 500 nM PbTx-3 (fig. 5B). The increase in $\tau_o$ was statistically significant at each membrane potential from $-40$ to 0 mV and saturated at PbTx-3 concentrations $\geq$500 nM. In the presence of PbTx-3, Na$^{+}$ channel openings are obtained at more negative potentials than control conditions, because of the shift in the activation curve.

**Effects and toxicity of various PbTx-3 derivatives.** To examine the effects of modification of the proposed active sites of the PbTx-3 molecule, the actions of the following PbTx-3 derivatives on single Na$^{+}$ channel activity were determined and compared with the naturally occurring toxin, 2,3-Dihydro-PbTx-3 A-ring diol (compound 3) was previously examined at various concentrations on single Na$^{+}$ channels (Baden et al., 1994). In the presence of 100 nM compound 3, only brief openings occurred immediately after the step depolarization, similar to those observed under control conditions (see fig. 2A). Within the concentration range examined, compound 3 (30–500 nM) had no effect on Na$^{+}$ channel activity. Raising the concentration of compound 3 to 1 $\mu$M caused a slight increase in Na$^{+}$ channel activity (n = 3; fig. 6A), whereas 10 $\mu$M compound 3 produced a substantial increase in Na$^{+}$ channel open probability (see fig. 4, Baden et al., 1994). The mean apparent open time was increased significantly at all voltages compared with control, and to the same degree as that observed with 100 nM PbTx-3 (fig. 2B). Compound 3 was approximately 10-fold less potent than PbTx-3, and concentrations $>10$ $\mu$M did not further increase the mean apparent open time. Under steady-state conditions, multiple subconductance levels of the open Na$^{+}$ channel were observed in the presence 10 $\mu$M compound 3 similar to those reported previously (data not shown; see Gawley et al., 1995).

Another PbTx-3 derivative with low toxicity is 2,3,27,28,41,43-hexahydro-PbTx-3 (compound 2). In the presence of compound 2, a pattern of Na$^{+}$ channel activity is observed that is similar to that seen with the same concentrations of compound 3 (fig. 6A). The low potency of both PbTx-3 derivatives is also evident by the concentration needed to modify the mean apparent open times (fig. 6B). Both compounds increase $\tau_o$ by 45 to 50% at all voltages compared with control conditions, but the mean apparent open times are less than those obtained in the presence of 500 nM PbTx-3 (broken line).

Figure 7A shows the effects of two other derivatives, PbTx-6 and 2,3,41,43-tetrahydro-PbTx-3 (compound 1), which were studied under similar conditions but at lower concentrations. The Na$^{+}$ channel activity evoked by 500 nM PbTx-6 or 500 nM compound 1 under steady-state conditions is higher than that observed with 1 $\mu$M compound 2 or 1 $\mu$M compound 3 (compare with fig. 6A), but less than that observed in the presence of 500 nM PbTx-3. Na$^{+}$ channels modified by PbTx-6 and compound 1 remain in the open conformation longer than the unmodified Na$^{+}$ channels (fig. 7B). The mean apparent open times for both derivatives are increased by $\geq$50% compared with control but are less than the calculated values for Na$^{+}$ channels modified by 500 nM.
PbTx-3, PbTx-6 and compound 1 also cause a shift in activation to more negative membrane potentials.

Discussion

The effects of brevetoxin-3 and its derivatives were studied in TTX-sensitive sodium channels in rat nodose sensory neurons. Previous studies of neonatal and adult vertebrate sensory neurons have described at least two different types of Na$^+$ currents according to their sensitivity to TTX: TTX-sensitive (TTX$_s$) and TTX-resistant (TTX$_r$). The two types of Na$^+$ currents exhibit different kinetics: one with fast kinetics that is blocked by TTX at nanomolar concentrations and the other exhibiting slower kinetics that is TTX-resistant (Campbell, 1993; Elliott and Elliott, 1993; Ikeda et al., 1986). Voltage-clamp studies in adult rat nodose ganglion cells (average diameter, 35 $\mu$m) similarly reveal both TTX-sensitive and TTX-resistant Na$^+$ currents. In the presence of 3 to 15 nM TTX, the TTX-sensitive Na$^+$ channels are blocked completely, whereas most of the TTX-resistant inward current persists (Ikeda et al., 1986). In contrast to neurons obtained from adult rats, neurons from the nodose ganglia of neonatal rats appear to express only one type of Na$^+$ channel which is blocked reversibly by 1 nM TTX. Complete block of Na$^+$ current at this relatively low TTX concentration indicates that the Na$^+$ inward current is caused exclusively by TTX-sensitive Na$^+$ channels.

The observed effects of PbTx-3 on voltage-dependent Na$^+$ channels in rat nodose ganglion neurons can be summarized as follows: 1) a shift of activation by $\pm$10 mV to more negative membrane potentials, 2) an inhibition of Na$^+$ channel inactivation at all membrane potentials whereby single Na$^+$ channel activity is observed during maintained depolarization, 3) an approximately 2-fold increase of the mean apparent channel open time at all membrane potentials and 4) the appearance of subconductance states with two distinct conductance levels detected (10.7 pS and 21.2 pS). These effects were observed from 30 to 500 nM PbTx-3. All effects of PbTx-3 observed in rat nodose neurons are consistent with those observed on Na$^+$ channels in rat cardiac myocytes (Schreibmayer and Jeglitsch, 1992) but differ from those observed in neuroblastoma cells (Sheridan and Adler, 1989).
The lack of effect of PbTx-3 on Na⁺ channel inactivation observed in neuroblastoma cells may partly be caused by the short-duration voltage pulses used to examine the kinetics of Na⁺ current inactivation in whole-cell and single-channel experiments.

All PbTx-3 derivatives examined caused a similar shift in activation to more negative membrane potentials, albeit at higher concentrations than found with PbTx-3. Similarly, inhibition of Na⁺ channel inactivation was observed for all PbTx-3 derivatives but to a lesser extent than that observed with PbTx-3. The mean apparent open times were increased by 30 to 50% compared with control by all PbTx-3 derivatives, but to a lesser degree than by PbTx-3. The effect of PbTx-3 derivatives on mean channel open time appears to be related to their binding affinity for the Na⁺ channel, whereby those derivatives with a low binding affinity exhibited a smaller effect (Rein et al., 1994). Subconductance states of the Na⁺ channel were also observed in the presence of PbTx-3 derivatives as reported previously in rat nodose neurons (Gawley et al., 1995). In the presence of PbTx-3, only two conductance levels were observed, whereas with 2,3-dihydro-PbTx-3 A-ring diol (compound 3), a minimum of five conductance levels were observed (Gawley et al., 1995).

The brevetoxin molecule has been shown to bind to receptor site 5 with the tail end of the molecule located near the S5-S6 extracellular loop of domain IV of the Na⁺ channel alpha subunit (Trainer et al., 1991, 1994). The finding that all derivatives of brevetoxin examined to date shift the voltage dependence of activation to more negative potentials indicates that the oxygen-rich nature of the brevetoxin backbone interacts with the channel in a manner that stabilizes the open configuration.

According to the model of the voltage-sensitive Na⁺ channel by Sato and Matsumoto (1995), all four extracellular loops formed by S5-S6 of all domains have to slide into the core pore formed by S2-S4 segments for full activation of the channel. In this case, the A-ring end of the PbTx-3 molecule may reach far enough into the membrane to interact with the charges of the inactivation gate and likely lead to inhibition of Na⁺ channel inactivation. In the presence of PbTx-3 and its derivatives, the open conformation is stabilized and the induction of subconductance states is observed in the toxin-

![Fig. 5. Voltage dependence of Na⁺ channel open time in the absence and presence of PbTx-3. (A) Unmodified (control) and modified (30 nM and 500 nM PbTx-3) single Na⁺ channel currents evoked by step depolarizations to −40 mV (lower current traces) and −10 mV (upper current traces) from a holding potential of −100 mV in cell-attached membrane patches. (B) The mean apparent open time (τₒ) obtained at different membrane potentials in the absence (Control, •) and presence of 30 nM (▼) and 500 nM (▼) PbTx-3. Each data point represents mean ± S.E. calculated from at least three experiments.](image-url)
modified Na$^+$ channel. The induction of subconductance states may be caused by the character of the A-ring and its proximity to the inactivation loop of the channel. This is consistent with results obtained with the truncated brevetoxin-B possessing all the “essential” chemical elements except length (20.4 Å compared with approximately 30 Å) (Nicolau et al., 1994) and the five conductance states induced by the 2,3-dihydro-PbTx-3 derivative (Gawley et al., 1995). The multiple subconductance states may be caused by the interaction of the “head” end of the toxin with the inactivation particle on the inside face of the channel associated with the loop between domains III and IV, which results in “partially open” (or partially closed) channels by differential physical occlusion. The potential for this physical misalignment of the inactivation particle is supported partly by computer modeling, which indicates a fixed angle and direction of orientation of the A-ring lactone in all closed ring toxins examined, and a general free range of movement for the “arms” of any A-ring-cleaved toxin (Rein et al., 1994). The mechanism(s) by which brevetoxins and derivatives generate the subconductance states remains to be elucidated, however.

The induction of longer mean open times is characteristic of all natural brevetoxins and synthetic derivatives, except the 1-deoxy-PbTx-3 derivative, which indicates that the carbonyl oxygen may be important for maintaining channels in the open configuration (Gawley et al., 1995). Given that voltage-dependent Na$^+$ channels have been proposed to activate and inactivate based on changing dipole pairs on adjacent $\alpha$-helices of channel domains (Catterall, 1992; Patlak, 1991), the effect of 1-deoxy-PbTx-3 toxin (which does not induce longer mean open times) is consistent with the importance of dipole interactions in brevetoxin stabilizing channels in the open configuration.

The overall length of the brevetoxin molecule may be the controlling factor in inhibition of inactivation once Na$^+$ channels have been activated. Again, results obtained with the 1-deoxy-PbTx-3 derivative indicate that an intact carbonyl moiety is not necessary for activity, whereas without the proper length (e.g., truncated brevetoxin), the presence of an intact A-ring is not sufficient to inhibit Na$^+$ channel inactivation (Gawley et al., 1995). The results of the present study have identified some of the salient features associated with polyether toxin modification of voltage-sensitive Na$^+$ chan-
nel gating and provide useful insights into the factors that modulate sodium channel function.

References


Fig. 7. Voltage dependence of Na\textsuperscript{+} channel open time in the presence of PbTx-6 and 2,3,41,43-tetrahydro-PbTx-3 (compound 1). (A) Modified (500 nM PbTx-6, 500 nM compound 1) single Na\textsuperscript{+} channel currents recorded from cell-attached membrane patches in response to step depolarizations to –40 mV (lower current traces) and –10 mV (upper current traces) from a holding potential of –100 mV. (B) The mean apparent open time (\(\tau_o\)) obtained at different membrane potentials in the absence (Control, □) and presence of 500 nM PbTx-6 (○) and 500 nM compound 1 (△). The dashed line indicates \(\tau_o\) values obtained in the presence of 500 nM PbTx-3. Each point represents mean ± S.E. calculated from at least three experiments.


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