Brevetoxin derivatives that inhibit toxin activity

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Background: The brevetoxins are marine neurotoxins that interfere with the normal functions of the voltage-gated Na^+ channel. We have identified two brevetoxin derivatives that do not exhibit pharmacological properties typical of the brevetoxins and that function as brevetoxin antagonists.

Results: PbTx-3 and benzoyl-PbTx-3 elicited Na^+ channel openings during steady-state depolarizations; however, two PbTx-3 derivatives retained their ability to bind to the receptor, but did not elicit Na^+ channel openings. α-Naphthoyl-PbTx-3 acted as a PbTx-3 antagonist but did not affect Na^+ channels that were not exposed to PbTx-3. β-Naphthoyl-PbTx-3 reduced openings of Na^+ channels that were not exposed to PbTx-3.

Conclusions: Some modifications to the brevetoxin molecule do not alter either the binding properties or the activity of these toxins. Larger modifications to the K-ring sidechain do not interfere with binding but have profound effects on their pharmacological properties. This implies a critical function for the K-ring sidechain of the native toxin.

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Introduction

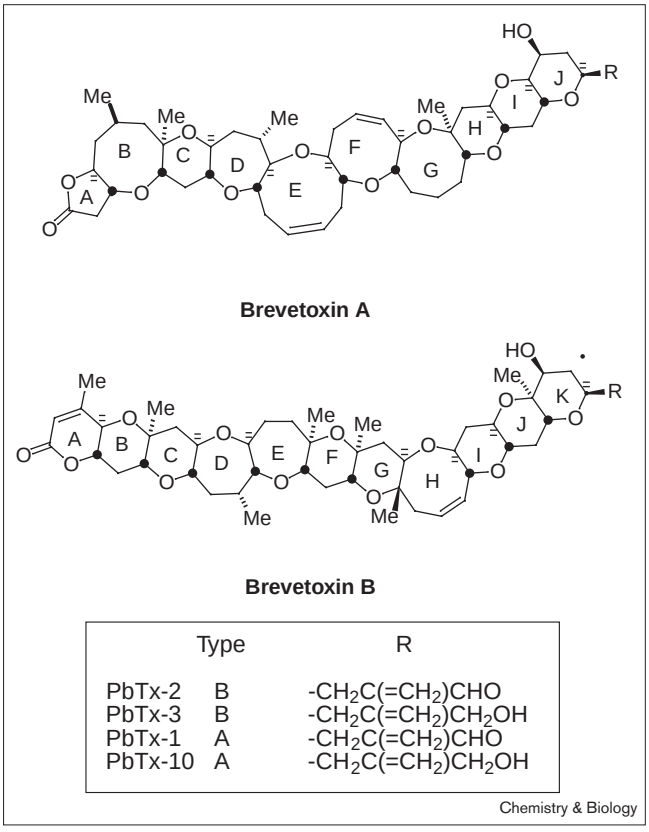
The brevetoxins are a series of structurally related neurotoxins produced by the 'red tide' dinoflagellate *Gymnodinium breve* [1]. Two skeletal types are known, each type having a number of congeners that vary in the K-ring (type B) or J-ring (type A) sidechains (Figure 1). These toxins are responsible for massive fish kills and marine mammal mortalities [2,3], primarily on Florida's Gulf Coast. Consumption of toxin-tainted shellfish induces a syndrome in humans known as neurotoxic shellfish poisoning. Symptoms include nausea, vomiting, diarrhea, bronchoconstriction, reversal of temperature sensation and parasthesias [4].

These effects are apparently brought about by the interaction of the toxins with a receptor site (site 5) on the voltage-gated Na^+ channel [1]. Specific activities include: a shift of activation potential for Na^+ channel opening to more negative values (i.e., channels open at normal resting potentials) [5,6]; inhibition of inactivation [5,6]; increased mean channel open times [5]; and induction of sub-conductance states [5,7]. Together these effects result in a dose-dependent depolarization of excitable membranes [8–10].

The functional α subunit of the voltage-gated Na^+ channel is composed of four highly homologous domains. Each domain includes six transmembrane α helices (S1–S6). The highly charged S4 α helices are thought to comprise the voltage-sensing functionality of the channel [11]. Photoaffinity labeling of the Na^+ channel has indicated that the specific brevetoxin-binding site, at least for the K-ring sidechain, is located near the S5–S6 extracellular loop of domain 4 [12]. The toxin is believed to orient itself across the membrane, parallel to the lipid bilayer and the α helices, with the K-ring sidechain end of the molecule pointing outward [13].

The evaluation of the binding properties of several natural and semisynthetic toxins, correlated with computer molecular modeling, has led to a 'common pharmacophore' hypothesis for site-5 ligands of the voltage-gated Na^+ channel. Briefly, it appears that both a critical length [14] and a specific conformation (relatively straight) [15,16] are required for toxin binding. The interactions between the toxin and its receptor site are believed to be mostly hydrophobic; however, the A-ring lactone provides enhanced stability to the ligand–receptor complex, presumably by acting as a

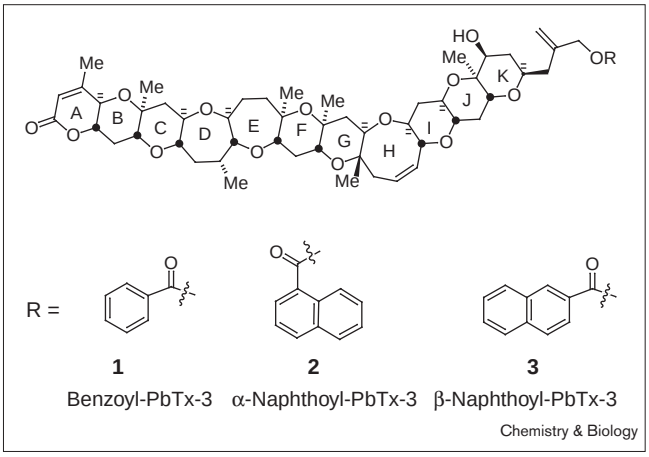
Figure 1



	Type	R
PbTx-2	B	$-CH_2C(=CH_2)CHO$
PbTx-3	B	$-CH_2C(=CH_2)CH_2OH$
PbTx-1	A	$-CH_2C(=CH_2)CHO$
PbTx-10	A	$-CH_2C(=CH_2)CH_2OH$

Structure of the two brevetoxin skeletal types and several congeners.

Figure 2



Structure of the brevetoxin derivatives evaluated in this study.

hydrogen-bond acceptor [17]. Interestingly, specific regions of the toxin molecule can be correlated with the four activities listed above. The relatively rigid H–K-ring region appears to possess characteristics of a requisite ligand-binding region, and the A-ring lactone is critical for inducing longer mean open times. The overall length of the toxin appears to deliver the lactone to an activity locus on the channel responsible for inhibition of inactivation; this is exemplified by the lack of this effect in a truncated form of brevetoxin possessing the binding H–K region and intact A-ring, but possessing a shorter spacer region (through deletion of rings B–E) [18].

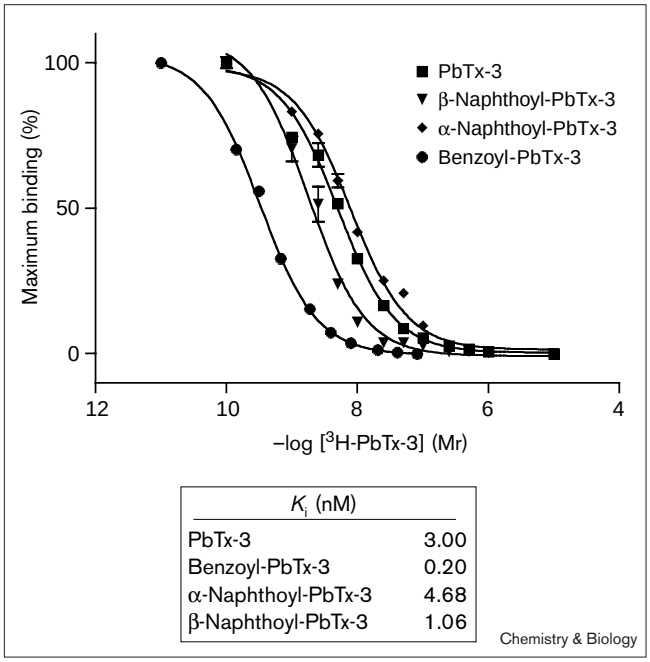
As part of a continuing effort to develop and refine the common pharmacophore hypothesis, we evaluated three new brevetoxin derivatives (Figure 2) in electrophysiological experiments. Our study builds on recent experiments [6] that examined the effects of PbTx-3 on single Na^+ channels in detail. Recordings from channels in individual membrane patches were made before and after toxin application. An advantage of this approach is that channels in an individual patch serve as their own control, thereby taking into account normal variability in Na^+ channel kinetics observed in membrane patches. Our comprehensive observations have led to the surprising conclusion that, although the three derivatives examined bind to site 5, two of them do not elicit any of the typical brevetoxin responses. Furthermore, at least one of these derivatives inhibits brevetoxin activity. In other words, our efforts have identified the first brevetoxin antagonist.

Results

The brevetoxin derivatives whose binding characteristics and physiological actions on Na^+ channels were evaluated in this study were benzoyl-PbTx-3 (**1**), α-naphthoyl-PbTx-3 (**2**) and β-naphthoyl-PbTx-3 (**3**; Figure 2). The binding affinities of the three derivatives were assessed in a competitive displacement assay (Figure 3). All three derivatives were able to displace ^{3}H-PbTx-3 from site 5 with nanomolar affinities. Thus, derivatization of the K-ring sidechain does not significantly interfere with the binding of the toxin to its receptor site. In fact, in two cases (**1** and **3**) binding is enhanced over that of PbTx-3. As a comparison, the site-1 toxin tetrodotoxin (TTX), which does not competitively displace site-5 toxins, was studied for its potential blocking actions of PbTx-3 in physiological assays.

Most subtypes of voltage-activated Na^+ channels open briefly and with a short latency in response to depolarizations within the Na^+ channel activation range from hyperpolarized holding potentials at which they are closed but not inactivated ('step depolarizations'). Furthermore, Na^+ channels inactivate during prolonged depolarizations without a return to a hyperpolarized potential and thus are normally not open during prolonged, steady-state depolarizations. In these experiments with B50 cells, the presence of normal Na^+ channel activity was confirmed by step-depolarization tests. In addition, a normal absence of channel activity was noted during steady-state

Figure 3



Competitive displacement assays for brevetoxin derivatives **1**, **2** and **3**, and PbTx-3.

depolarizations lasting 7.2 s of continuous observation. Consistent with previous studies using nodose ganglion [5] and B50 and B104 cell lines [6], the most notable effect of PbTx-3 was its ability to open Na^+ channels in B50 membrane patches during steady-state depolarizations lasting many seconds.

Unitary Na^+ currents in the steady state are illustrated in Figure 4. The presence of Na^+ channels in the membrane patches was confirmed during step-depolarization trials performed before the steady-state experiment of Figure 4a and b, and no Na^+ channel openings were observed during steady-state depolarizations in the absence of toxin (Figure 4a; control). PbTx-3 application to the bath elicited Na^+ channel openings to the full open amplitude (Figure 4b) and also to substates [5] during steady-state depolarizations (see first trace of Figure 4b). Figure 4 also demonstrates the effects of prior exposure to TTX on PbTx-3-elicited steady-state Na^+ channel openings. TTX was added to the bath before the membrane seal was formed (Figure 4c), and then PbTx-3 was added (Figure 4d). No PbTx-3-induced channel openings were observed in the presence of TTX ($n = 5$) during periods ranging from 7.2 to 9 s of continuous observation. Thus, although TTX and PbTx-3 act at different sites on the Na^+ channel molecule, TTX blocks PbTx-3-induced effects on steady-state channel openings.

In an effort to refine our common pharmacophore hypothesis and to correlate structural features with physiological consequences, we examined the effects of PbTx-3 derivatives on Na^+ channels. Figure 5 illustrates unitary Na^+ currents in the steady state in the presence of PbTx-3 or one of three derivatives of PbTx-3. During step depolarizations these membrane patches were observed to contain

Figure 4

Steady-state Na^+ channel currents in two different membrane patches recorded at a continuous voltage equal to the command potential of −50 mV (top) or −10 mV (bottom) plus the cell's unknown resting potential. The Na^+ current activation voltage observed in whole-cell current–voltage experiments [6] estimates the cell resting potentials in this and subsequent experiments in normal extracellular solution (ECS; see the Materials and methods section) at −20 to −10 mV. Traces illustrate a membrane patch **(a)** before and **(b)** after exposure to PbTx-3 (100 nM, 20 KHz sampling rate, $n = 50$) or a membrane patch exposed first **(c)** to 100 nM TTX and then **(d)** to 100 nM PbTx-3 (100 nM, 50 kHz, $n = 5$). No channel openings were observed in TTX at command potentials between −50 and −10 mV. Although it was not possible to determine that the membrane patches of TTX-exposed cells contained Na^+ channels, we estimate that the probability that these five membrane patches lacked Na^+ channels is very low because under our culture conditions only 2% of membrane patches from B50 cells lack functional Na^+ channels.

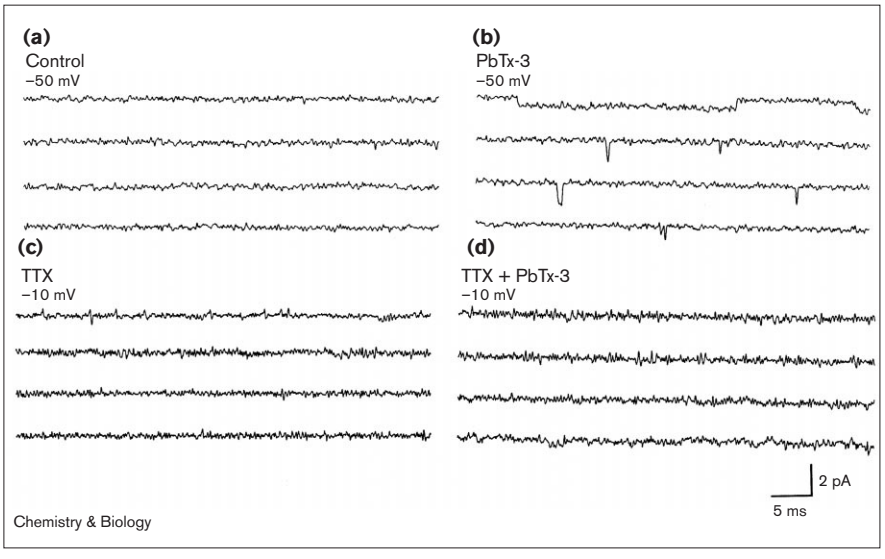
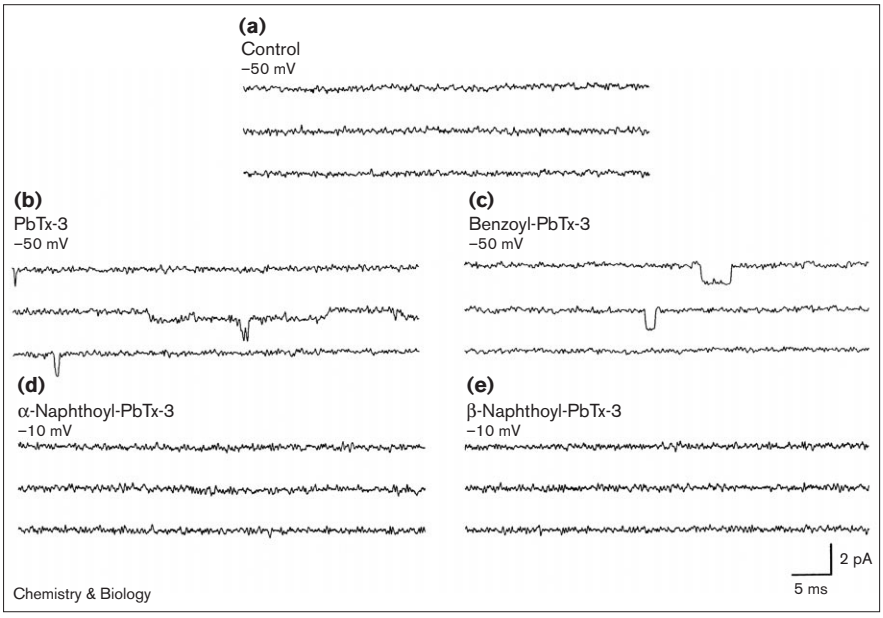


Figure 5



Steady-state currents in four different membrane patches at a steady-state command voltage of –50 mV. **(a)** Control traces preceded the test with PbTx-3, and are representative of control recordings observed in all four patches during 7.2 s of continuous observation. Membrane patches were exposed to **(b)** PbTx-3 (100 nM, $n = 50$) or **(c,d,e)** 1 of 3 derivatives of PbTx-3 (benzoyl-PbTx-3, $n = 3$, α-naphthoyl-PbTx-3, $n = 8$; or β-naphthoyl-PbTx-3, $n = 6$, respectively; all 100 nM). The experimental recordings are representative of 7.2 s of continuous observation in PbTx-3 or the indicated derivative.

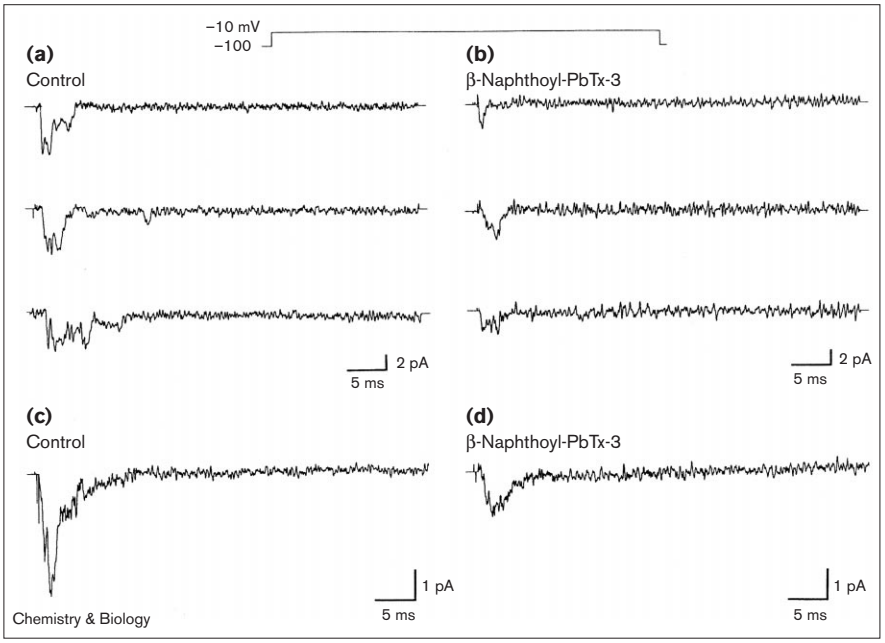
normally behaving Na^+ channels, and no Na^+ channel openings were observed during steady-state depolarizations in the absence of toxin (Figure 5a; control). PbTx-3 application to the bath elicited substate and full open amplitude Na^+ channel openings (second and third traces of Figure 5b, respectively). In separate membrane patches, following control observations, the PbTx-3 derivative benzoyl-PbTx-3 elicited Na^+ channel openings in the steady state (Figure 5c), but α-naphthoyl-PbTx-3 and β-naphthoyl-PbTx-3 did not (Figure 5d and e). Whereas some modifications (such as benzoyl) of the PbTx-3 molecule do not result in loss of effectiveness in eliciting steady-state openings [6], the larger naphthoyl groups appear to abolish the ability of the toxin molecule to elicit steady-state openings. This raises the question of whether the larger naphthoyl groups are blocking the channel.

We studied the effects of α- and β-naphthoyl-PbTx-3 on Na^+ channel openings during successive depolarizations lasting 50 ms to –10 or –20 mV from a resting command potential of –100 mV in order to test the ability of these derivatives to block unmodified (control) Na^+ channels. During depolarizing pulse experiments, α-naphthoyl-PbTx-3 did not block Na^+ channels ($n = 10$) and did not induce a shift in the threshold for channel opening ($n = 3$; data not shown). The actions of β-naphthoyl-PbTx-3 during 50 ms step depolarizations to –10 mV are summarized in Figure 6. Figure 6a and b represents recordings before and after β-naphthoyl-PbTx-3 treatment, respectively. Figure 6c and d are the ensemble averages of 24 50 ms depolarizations under these conditions. In the control period, most Na^+ channel openings occurred during the first 5 ms of the depolarizing pulse but later openings were occasionally observed. After the addition of 100 nM β-naphthoyl-PbTx-3 to the bath, fewer Na^+ channel openings occurred, and few openings occurred later than 5 ms after the beginning of the pulse, which is unlike the late channel-opening pattern that is characteristic of PbTx-3 [6]. These results suggest that channel activation and re-opening were inhibited by β-naphthoyl-PbTx-3. The time course of macroscopic inactivation of the ensemble-averaged currents (τ_h) was fit by a single exponential, and the areas under the fitted curves were calculated. The area was 11.1 pA × ms before toxin application (Figure 6c) and 1.29 pA × ms after toxin application (Figure 6d), a decrease of 88% in this experiment. In ensemble averages from six patches consisting of depolarizations to –10 or –20 mV, the mean area of the controls was 32.68 ± 18.2 pA × ms and that after toxin exposure was 9.23 ± 6.88 pA × ms, an average of 71.8% less after exposure to β-naphthoyl-PbTx-3. Control area averages were significantly different from β-naphthoyl-PbTx-3-exposed area averages in the same membrane patches (paired t-test, $p \leq 0.008$).

An additional feature of the actions of β-naphthoyl-PbTx-3 during brief depolarizations was that it induced a greater than 5 mV hyperpolarizing shift in the activation threshold (in 2 out of 2 patches; data not shown). This shift is an independent consequence of the apparent inhibitory effect of this derivative on Na^+ channel openings.

The potential for α- and β-naphthoyl-PbTx-3 to act as PbTx-3 antagonists is suggested by three results: the

Figure 6



Single Na^+ channel currents during successive command depolarizations to −10 mV from a command potential of −100 mV in the same membrane patch **(a)** before and **(b)** after exposure to β-naphthoyl-PbTx-3 (100 nM, $n = 6$). **(c,d)** Ensemble averages indicated a reduction in peak Na^+ current. The average area under the β-naphthoyl-PbTx-3 curve was 71.8 ± 18.3% less than that of the control.

apparent ability of α- and β-naphthoyl-PbTx-3 to displace the native toxin from its binding site; the lack of steady-state channel activity in the presence of α- and β-naphthoyl-PbTx-3; and the inhibition of Na^+ channels by β-naphthoyl-PbTx-3. To test the possible PbTx-3 antagonist effects of α-naphthoyl-PbTx-3, we investigated the interactions between PbTx-3 or benzoyl-PbTx-3 and α-naphthoyl-PbTx-3. Figure 7 shows the steady-state effects of addition of α-naphthoyl-PbTx-3 to the bath several minutes after addition of PbTx-3 to the bath. α-Naphthoyl-PbTx-3 abolished Na^+ channel activity induced in the steady state by PbTx-3 (Figure 7b,c). Figure 8 shows that some blocking effects of α-naphthoyl-PbTx-3 extend to benzoyl-PbTx-3, which has the highest binding affinity of the group. Benzoyl-PbTx-3 was added to the bath after control records were made (Figure 8a,b), and the cell was additionally exposed to α-naphthoyl-PbTx-3 (Figure 8c). Benzoyl-PbTx-3-induced channel openings were blocked by further addition of α-naphthoyl-PbTx-3 to the bath, although occasional, brief Na^+ channel openings were observed (third trace of Figure 8c). In the membrane patch shown, Na^+ channels were open for 12.8 ms of each second of observation time in benzoyl-PbTx-3, whereas after addition of α-naphthoyl-PbTx-3 channels were open for 0.37 ms of each second.

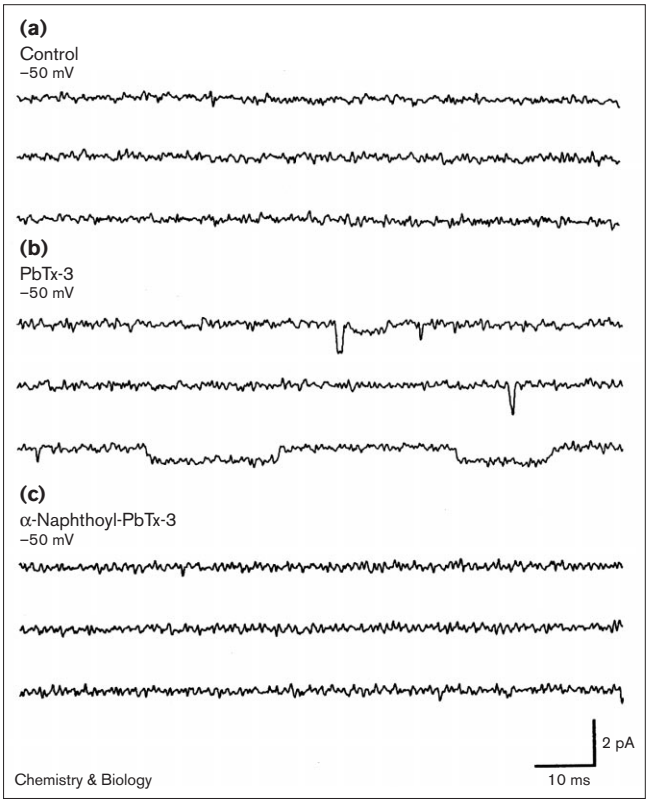
The lack of any demonstrable physiological effect elicited by α-naphthoyl-PbTx-3 prompted us to evaluate the toxicity of this derivative in a fish bioassay. The concentration required to kill 50% of the fish (LC_{50}) for this compound was determined to be 142 nM in a 24 h assay. This is in contrast to an LC_{50} of 15 nM for PbTx-3.

Discussion

The pharmacological interactions between TTX and PbTx-3 illustrate a unique comparison of the competition between site-1 and site-5 Na^+ channel toxins. Although TTX and PbTx-3 act at different sites on the Na^+ channel molecule, the effects of TTX preclude those of PbTx-3 regardless of the order in which these toxins are added to membrane patches. Thus, if TTX is applied first to membrane patches containing Na^+ channels, it blocks PbTx-3-induced effects on steady-state channel openings. Conversely, if PbTx-3 is applied first, eliciting steady-state Na^+ channel openings, subsequent application of TTX blocks them [6]. Therefore, TTX-induced channel block at site 1 takes precedence physiologically, regardless of 'competing' PbTx-3-induced modulation of channel opening occurring at site 5.

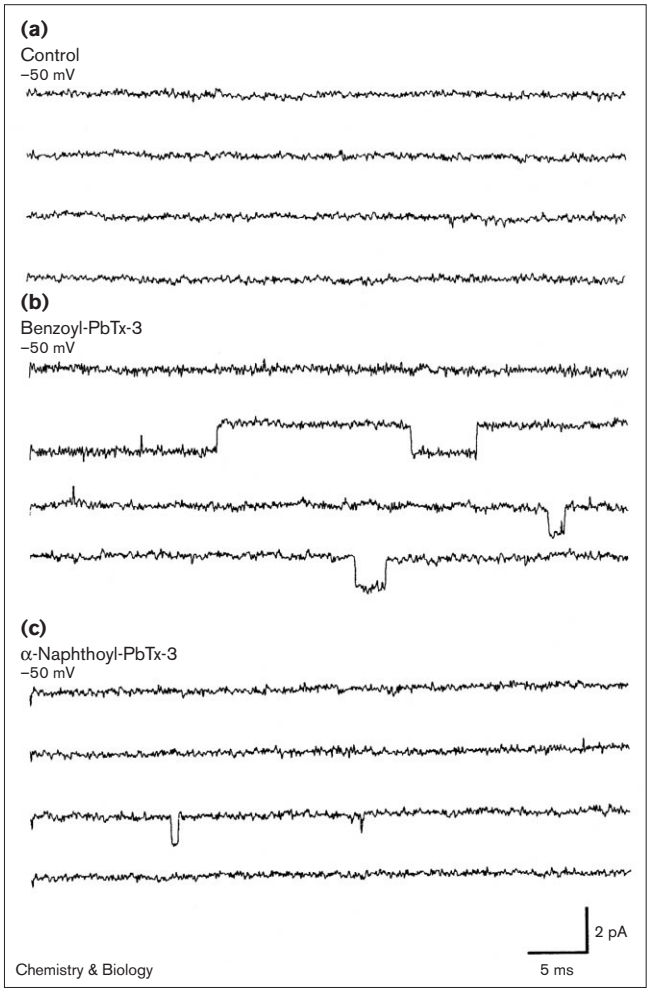
Table 1 summarizes the results of the PbTx-3 binding and single-channel experiments. All three PbTx-3 derivatives bind site 5 of the voltage-gated Na^+ channel and have physiological activities with some relationship to native PbTx-3. Benzoyl-PbTx-3 is similar to native PbTx-3 in its ability to elicit Na^+ channel openings in the steady state. These openings, like those elicited by native PbTx-3, lasted tens of milliseconds, and occurred more frequently after the first 5 ms of depolarization, suggesting that channel inactivation had been slowed. Although a full characterization of the effects of benzoyl-PbTx-3 was not

Figure 7



Steady-state currents recorded in normal ECS at a continuous command voltage of −50 mV **(a)** before and after exposure to **(b)** PbTx-3 (100 nM) and then **(c)** α-naphthoyl-PbTx-3 (100 nM, $n = 5$). The frequency of channel opening in the control was 0 ms/s; after PbTx-3, 12.8 ms/s; and after the addition of α-naphthoyl-PbTx-3, 0.02 ms/s.

Figure 8



Steady-state currents recorded in normal ECS at a continuous command voltage of −50 mV **(a)** before and after exposure to **(b)** benzoyl-PbTx-3 (100 nM) and then **(c)** α-naphthoyl-PbTx-3 (100 nM, $n = 2$). The frequency of channel opening in the control was 0 ms/s; after benzoyl-PbTx-3, 12.8 ms/s; and after the addition of α-naphthoyl-PbTx-3, 0.37 ms/s.

made, it appears that the relatively small modification of the PbTx-3 sidechain made in the benzoyl derivative does not modify the actions of the PbTx-3 molecule to any significant extent.

In contrast to benzoyl-PbTx-3, α-naphthoyl-PbTx-3 and β-naphthoyl-PbTx-3 did not open Na^+ channels in the steady state when applied alone (Figure 4). Thus, modification of the PbTx-3 sidechain with large naphthoyl groups abolished the most characteristic physiological activity of PbTx-3, despite binding constants that indicate these modifications do not interfere with the derivatives' binding to the appropriate site on the Na^+ channel molecule.

β-Naphthoyl-PbTx-3 inhibited Na^+ channel openings in test depolarization experiments, whereas α-naphthoyl-PbTx-3 blocked PbTx-3- and benzoyl-PbTx-3-induced Na^+ channel activity in steady-state experiments. Thus, large modifications of the PbTx-3 sidechain markedly changed the physiological effects of PbTx-3 from enhancement of Na^+ channel openings [5,6] to both block of unmodified Na^+ channels (β-naphthoyl-PbTx-3) and block of Na^+ channels activated by PbTx-3 or benzoyl-PbTx-3 (α-naphthoyl-PbTx-3). The effectiveness of α-naphthoyl-PbTx-3 as a PbTx-3 antagonist is interpreted as an observable physiological consequence of the displacement of PbTx-3 from its binding site by α-naphthoyl-PbTx-3. However, occasional, 'brief' Na^+ channel openings were observed (third trace of Figure 8c) when benzoyl PbTx-3 is the activator. This residual activity may be a consequence of the enhanced affinity of benzoyl-PbTx-3 (K_i = 0.2 nM) over PbTx-3 (K_i = 3.0 nM) for site 5 of the Na^+ channel.

The physiological effects of β-naphthoyl-PbTx-3 are considerably more complex than those of the α derivative. The voltage dependence of Na^+ channels means that channels

Table 1

Summary of effects of PbTx-3 and its derivatives.

	PbTx-3	Benzoyl PbTx-3	α-Naphthoyl-PbTx-3	β-Naphthoyl-PbTx-3
K_i (nM)	3.0	0.2	4.68	1.06
Promotes steady state Na^+ channel openings	Yes	Yes	No	No
Block of unmodified Na^+ channels	No	nd	No	Yes
Inhibits PbTx-3 or benzoyl PbTx-3-induced steady-state openings	–	–	Yes	nd
Hyperpolarized shift in Na^+ channel activation curve	6.7 mV*	No	No	~5 mV

*From [6]. nd, not determined.

open only sporadically at voltages near the activation threshold and that channel-opening activity is greater at voltages more depolarized than this threshold. A shift in the Na^+ channel activation threshold toward more hyperpolarized voltages, as observed upon exposure of membrane patches to β-naphthoyl-PbTx-3, is an independent consequence of the apparent inhibitory effect of this derivative on Na^+ channel openings. A hyperpolarizing shift alone would increase channel activity at voltages near the peak of the voltage-activation range. Instead, a significant decrease in channel opening was observed in the presence of β-naphthoyl-PbTx-3. Thus, β-naphthoyl-PbTx-3 exerts two contradictory effects on channel gating: lowering of the threshold for activation, and apparent channel block.

Although the mechanism of Na^+ channel block by β-naphthoyl-PbTx-3 is unclear, the mode of action of the brevetoxins is believed to be through the lipid border of the channel and not from the pore surface [6]. This is in contrast to a site-1 blocker such as TTX which acts through channel occlusion.

One interpretation of these results is that PbTx-3 lowers the activation threshold by lowering the activation barrier to the conformational change that occurs when channels shift from the closed to the open state. At the same time PbTx-3 increases mean channel open times by stabilizing the open conformation relative to the closed and inactivated conformations. The β-naphthoyl derivative also lowers the activation barrier between the closed and open states, but it destabilizes the open state relative to the closed or inactivated states, which results in apparent inhibition of channel opening. The destabilizing actions of β-naphthoyl-PbTx-3 may result in a decrease in the channel open time, but this was not determined here. As a result, the data presented for β-naphthoyl-PbTx-3 cannot rule out channel occlusion. It is possible that the bulky sidechain might penetrate the pore while the toxin is strategically placed at site 5.

Photoaffinity labeling of the brevetoxin binding site using a *p*-azidobenzoyl-PbTx-3 derivative has indicated the the *p*-azido group is located near the S5–S6 extracellular loop on domain 4 of the voltage-dependent Na^+ channel. That a synthetic modification to the portion of the molecule that interacts with this loop should abolish all activity is intriguing and suggests that this portion of the α subunit and the K-ring sidechain of brevetoxin have a critical function. This loop contains the α-scorpion toxin binding site (part of site 3). Binding of toxin is strongly voltage dependent, indicating that this site may be involved in channel activation and inactivation [11]. An important role for the K-ring sidechain in brevetoxin has previously been suggested [19]. Conformational analysis of the sidechains of native and derivative toxins reveals no obvious differences in conformational preferences. The global minimum for the native toxin places the sidechain methylene away from the K-ring with the hydroxyl group positioned over the ring by a hydrogen bond to the K-ring ether oxygen. One might imagine that this orientation would be increasingly difficult to achieve as steric bulk of the sidechain increased. This appears not to be the case for any of the derivatives **1**, **2** or **3**, however, as all three have very similar global minima. Other conformations placing the sidechain away from the toxin are equally accessible. Furthermore, as binding is unaffected it seems unlikely that a conformational change is responsible for the observed electrophysiological effects. It seems more likely that the brevetoxin skeleton serves to anchor the toxin sidechain into the proper position for a crucial interaction with a portion of the α subunit (possibly the S5–S6 extracellular loop of domain 4). Large groups such as α- and β-naphthoyl derivatives may not interfere with binding but do disrupt this interaction. This hypothesis could be tested by evaluating a semisynthetic toxin that lacks a sidechain.

Although α-naphthoyl-PbTx-3 behaves as a brevetoxin antagonist *in vitro*, *in vivo* it acts as a toxin with an LC_{50} of 142 nM. All other brevetoxin derivatives evaluated exhibit

a positive correlation between the inhibitor concentration required to displace 50% of the specific binding of the radiolabeled ligand (IC_{50}; from the receptor binding assay) and LC_{50}. On the basis of the IC_{50}, we could expect an LC_{50} roughly equivalent to that of PbTx-3 (15 nm) [15]. We cannot rule out another mechanism of activity, but we suspect that the toxicity was due either to the activity of an esterase cleaving the α-naphthoyl derivative to PbTx-3 or metabolic conversion to another active metabolite. The preparation of a structurally similar derivative with a less labile linkage could provide an antagonist that acts both *in vitro* and *in vivo*.

Significance

The brevetoxins are polyether ladder molecules produced by the 'red tide' dinoflagellate *Gymnodinium breve* that induce a syndrome known as neurotoxic shellfish poisoning when consumed in tainted shellfish. These and other dinoflagellate-derived polyethers cause severe and sometimes fatal neurological dysfunction after consumption of contaminated seafood. Blooms of these toxin-producing organisms can cause massive fish kills and marine mammal mortalities. The production and distribution of dinoflagellate-derived polyethers can therefore have severe and deleterious impacts on public health, environmental resources and local economies. Polyether ladder toxins are involved in 'red tide' and incidences of shellfish poisonings worldwide and the frequency and distribution of red tides appear to be increasing.

Efforts to counteract the deleterious effects of these potent neurotoxins should begin with a precise understanding of the molecular interactions required for toxicity. These molecules interact with the voltage-gated Na^+ channel, altering the properties of excitable membranes. This study expands on the 'common pharmacophore' hypothesis, which strives to identify the salient molecular features required for receptor binding and activity. This study has revealed that small modifications to the K-ring sidechain of the brevetoxin molecule have little effect on binding to the receptor site or pharmacological activity; however, bulky groups (α-naphthoyl and β-naphthoyl) do not inhibit binding but have a profound influence on activity. This suggests a crucial role for the K-ring sidechain in eliciting pharmacological effects. A more thorough evaluation of this crucial interaction could lead to a better understanding of the mechanism of neurotoxicity. Not only can these derivatives serve as a molecular probes but their ability to act as antagonists suggests possible opportunities for the development of therapies for intoxicated individuals.

Materials and methods

Dinoflagellate cultures

Unialgal cultures of *Gymnodinium breve* were grown in 10 l carboys in Wilson's NH15 media. The temperature was maintained at 20°C under continuous illumination from Grow-Lux Wide Spectrum fluorescent lamps at an incident illumination of 400 Lux.

Toxin purification and characterization

Brevetoxins were purified from laboratory cultures by a combination of chloroform/methanol extraction and thin-layer chromatography [1]. PbTx-3 was routinely purified by high performance liquid chromatography (HPLC; 85% isocratic methanol) using a Microsorb-MV, C-18 column (5 μM, 25 cm bed) and monitored by UV at 215 nM. ^{1}H-NMR spectra were recorded in $CDCl_3$ ($CHCl_3$ internal standard) at 400 MHz. Mass spectra were run in either desorption chemical ionization (DCI) or fast-atom bombardment (FAB) mode. High-resolution mass spectra were obtained from the mass spectrometry facility at the University of California, Riverside.

Synthesis of toxin derivatives

A tenfold excess (relative to PbTx-3) of carbonyl diimidazole and the corresponding acid (benzoic, α-naphthoic or β-naphthoic) were combined under nitrogen, at room temperature, in dry benzene. The solution was stirred for 30 min and then added to PbTx-3 in a 5 ml reaction vial. The reaction vial was sealed and the mixture was stirred overnight at 80°C. The reaction mixture was washed with an equal volume (3×) of saturated sodium bicarbonate, an equal volume (3×) of 10% HCl and evaporated under vacuum. The residue was purified using HPLC.

*Benzoyl-PbTx-3 (**1**).* Diagnostic peaks in the ^{1}H NMR include δ7.44 (2H, t, J = 7.2 Hz), 7.56 (1H, t, J = 7.2 Hz), 8.061 (2H, d, J = 8.4 Hz), 4.81 (2H, dd, J = 5.2 Hz) (C42). The C42 methylene is typically shifted downfield from its position in PbTx-3, and these diastereotopic protons are split into a doublet of doublets in the esters, whereas they appear as a singlet in PbTx-3. DCI MS (NH_3): 1002 (M + 1). HRMS (FAB): calc'd for $C_{57}H_{77}O_{15}$ (MH^+), calc'd 1001.5262, found 1001.5287.

*α-Naphthoyl-PbTx-3 (**2**).* Diagnostic peaks in the ^{1}H NMR include δ8.93 (1H, d, J = 8.8 Hz), 8.23 (1H, d, J = 8.8 Hz), 8.03 (1H, d, J = 8.8 Hz), 7.83 (1H, d, J = 8.8 Hz), 7.64 (1H, t, J = 8.8 Hz), 7.44 (2H, m), 4.92 (2H, dd, J = 5.2 Hz) DCI MS (NH_3): 1052 (M + 1). HRMS (FAB): calc'd for $C_{61}H_{79}O_{15}$ (MH^+), calc'd 1051.5419, found 1051.5367.

*β-Naphthoyl-PbTx-3 (**3**).* Diagnostic peaks in the ^{1}H NMR include δ8.64 (1H, s), 7.95 (3H, m), 7.58 (3H, m) 4.89 (1H, s).

Receptor-binding experiments

Synaptosomes were prepared by the method of Dodd *et al.* [20] from the whole brains of male Sprague–Dawley rats. Total binding was measured using a previously described rapid centrifugation technique [15]. IC_{50} data were obtained by fitting a one-site competition curve to displacement data using the program Prism (v 1.0, Graph Pad, Inc.). Although a detailed analysis of binding parameters was not performed, binding was assumed to be competitive by analogy with p-azidobenzoyl-PbTx-3 and other brevetoxin derivatives [12,15]. Inhibition constants (K_is) were calculated by the method of Cheng and Prussoff [21].

Computational methods

Internal coordinate Monte Carlo sampling, minimization using the MM2* force field and structure comparisons were performed using MacroModel (v. 6.5) and Batchmin (v. 6.5) [22]. Dihedral angles of the K-ring sidechain were varied randomly within a range of 0 to ± 180°. Minimizations were performed using the truncated Newton Raphson algorithm with a maximum of 2000 iterations, a derivative convergence of 0.01 kJ/(Å-mol), and a large (50 kJ/mol) energetic window.

Cell culture

The B50 cell line, subcultured from an original culture provided by D. Schubert (Salk Institute, San Diego, CA), was maintained as previously described [6]. The B50 cell line expresses types I and II Na^+ channel mRNAs [23].

Icthyotoxicity

Icthyotoxicity was assessed in a 24 h assay using mosquito fish, *Gambusia affinis*, as previously described [15].

Electrophysiological recording and analysis

Single Na^+ channel recordings were made using the cell-attached configuration of the patch-clamp technique, using thick-walled borosilicate pipettes (WPI, Inc., Sarasota, FL, USA) of 3–6 MΩ coated with Sylgard (Dow Corning Corp., Midland, MI, USA) to reduce electrode capacitance. Cell-attached single-channel recordings were made with an Axopatch 1D and digitized by a Labmaster TL-1 converter (both from Axon Instruments, Foster City, CA, USA) to a 486-33 MHz IBM-compatible computer using the PClamp6 Programs (Axon Instruments). Recordings made at room temperature were digitized at 50 kHz unless otherwise stated, and lowpass filtered at 2 kHz.

The bath solution (ECS) for single-channel recordings contained (in mM): 160 NaCl, 3 KCl, 1 $CaCl_2$, 0.6 $MgCl_2$, 7 D-glucose and 10 *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]-(HEPES-)NaOH, pH 7.4. The pipette solution consisted of ECS with additions of 30 mM Et_4NCl, 2.5 mM $BaCl_2$ and 10 μM $CdCl_2$ (to block K^+ and Ca^{2+} channels).

Aqueous stock solutions of PbTx-3 and PbTx-3 derivatives were prepared from concentrated solutions in 95% ethanol before addition to test dishes. Toxin was applied to cells by pipetting several microlitres of toxin in a bolus dose into the edge of the bath far from the cell under study. The final concentration of ethanol was 0.01%.

Assessment of the actions of PbTx-3 or its derivatives on single Na^+ channels was made using a recently described protocol [6]. Channel activity was recorded first in the absence of toxin (control). PbTx-3 or one of its derivatives was applied to the bath surrounding each membrane patch. Because of the lipophilic nature of PbTx-3, the toxin reached Na^+ channels in the membrane patch by lateral diffusion in the cell membrane. Channel activity was recorded 30 s after toxin application. Tetrodotoxin solutions (TTX, Research Biochemicals International, Natick, MA, USA) were prepared fresh daily. Unless otherwise mentioned, reagents were purchased from Sigma (St. Louis, MO, USA).

Single-channel records were analyzed as previously described [6] using the PClamp6 programs. The voltages associated with single-channel data represent the negative of the voltage applied to the membrane patch by the amplifier, referred to as the command potential. These voltages represent hyperpolarizations of the patch membrane [24]. It must be noted, however, that the actual patch potential equals the sum of the command potential and an unknown resting potential. Data are reported as mean ± SEM unless otherwise stated. Differences between control and toxin-treated channels were assessed using parametric statistical tests.

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