The relationship of brevetoxin 'length' and A-ring functionality to binding and activity in neuronal sodium channels

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Background: Brevetoxins are polyether ladder toxins that are ichthyotoxic at nanomolar concentrations. They bind to voltage-gated sodium channels, causing four distinct electrophysiological effects: (i) a shift of activation potential; (ii) occurrence of subconductance states; (iii) induction of longer mean open times of the channel; and (iv) inhibition of channel inactivation. We set out to determine whether these functions all require the same structural elements within the brevetoxin molecules.

Results: Several synthetically prepared structural analogs of brevetoxin B were examined in synaptosome receptor binding assays and by functional electrophysiological measurements. A truncated analog is not ichthyotoxic at micromolar concentrations, shows decreased receptor-binding affinity, and causes only a shift of activation potential without affecting mean open times or channel inactivation. An analog with the A-ring carbonyl removed binds to the receptor with nanomolar affinity, produces a shift of activation potential and inhibits inactivation, but does not induce longer mean open times. An analog in which the A-ring diol is reduced shows low binding affinity, yet populates five subconductance states.

Conclusions: Our data are consistent with the hypothesis that binding to sodium channels requires an elongated cigar-shaped molecule, ~30 Å long. The four electrophysiological effects of the brevetoxins are not produced by a single structural feature, however, since they can be decoupled by using modified ligands, which are shown here to be partial sodium channel agonists. We propose a detailed model for the binding of brevetoxins to the channel which explains the differences in the effects of the brevetoxin analogs. These studies also offer the potential for developing brevetoxin antagonists.

Introduction

Brevetoxin B (PbTx-2, Fig. 1), isolated from the Florida red tide dinoflagellate *Gymnodinium breve* (*Ptychodiscus brevis*) is one of several polyether ladder toxins that bind to the orphan receptor known as 'site 5' on the α subunit of voltage-gated sodium channels [1]. Other toxins that bind at the same site, shown by displacement of radioactive toxin in a competitive manner, include PbTx-3, 5, 6, 8, and 9 (Fig. 1), all of which have the same carbon skeleton (backbone) as PbTx-2. Two toxin classes with different polyether ladder backbones, brevetoxin A and ciguatoxin, also compete with brevetoxin B for binding to voltage-gated sodium channels. The structural similarities between these three classes of molecules are somewhat limited: all three toxins have trans-fused polycyclic ring systems composed of a single carbon chain that winds the length of the molecule, with only occasional 1-carbon 'branching.' In each toxin structure, the chain is linked by ether oxygens into a

series of *trans/syn* fused polycyclic arrays, with ring sizes ranging from five- to nine-membered.

The α subunit of the voltage-gated sodium channel is thought to comprise 24 transmembrane helices grouped into four domains (I–IV), each of which is divided into six subdomains (S1–S6), as shown in Figure 2. A number of conformations of the channel protein have been identified (or postulated), which explain the normal function of the channel [2–4]. These conformations are grouped into three functionally distinct (and presumably also allosterically distinct) classes: open, closed, and inactivated. Only the open conformation is conducting, and the closed conformation is the resting state. After activation by step depolarization (closed → open), the channel normally goes to an inactivated non-conducting state before returning to the closed, or resting state. A three-residue hydrophobic motif (Ile–Phe–Met) within a cytoplasmic loop of the channel polypeptide (referred to as the IFM
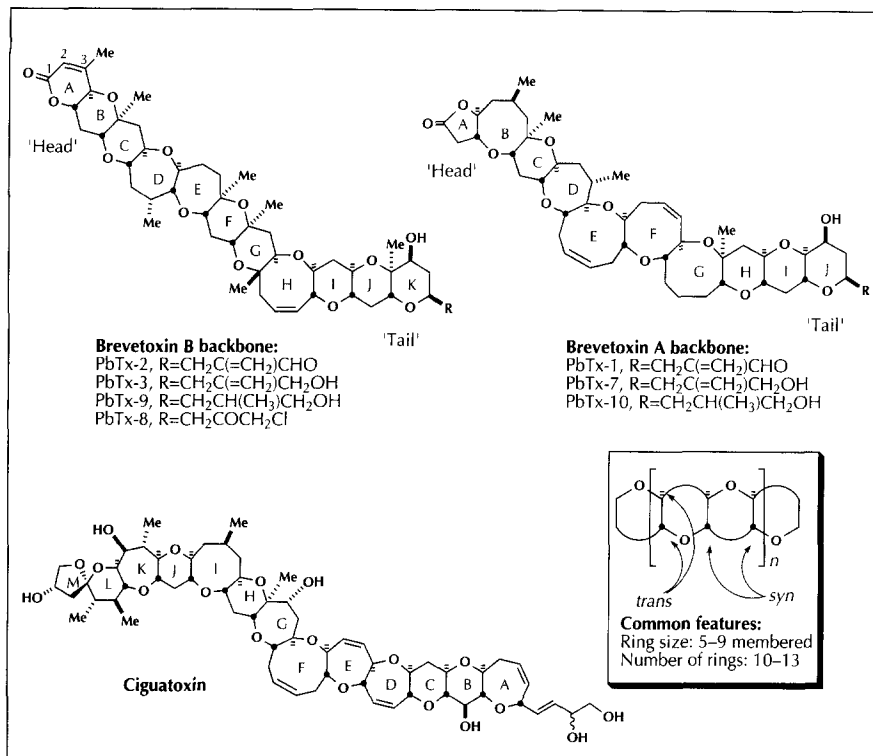
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Fig. 1. Structures of the three classes of natural toxins that bind to site 5 of the voltage-gated sodium channel. The common features of all three toxin classes are summarized in the box. Derivatives of PbTx-2 are the K-ring acetate, PbTx-5, and the H-ring epoxide, PbTx-6.

Brevetoxin B backbone:
PbTx-2, R=CH$_2$C(=CH$_2$)CHO
PbTx-3, R=CH$_2$C(=CH$_2$)CH$_2$OH
PbTx-9, R=CH$_2$CH(CH$_3$)CH$_2$OH
PbTx-8, R=CH$_2$COCH$_2$Cl

Brevetoxin A backbone:
PbTx-1, R=CH$_2$C(=CH$_2$)CHO
PbTx-7, R=CH$_2$C(=CH$_2$)CH$_2$OH
PbTx-10, R=CH$_2$CH(CH$_3$)CH$_2$OH

Ciguatoxin

Common features:
Ring size: 5–9 membered
Number of rings: 10–13

particle, see Fig. 2) is an essential structural feature of the channel, and may function as an inactivation lid that blocks ion conduction.

When brevetoxin B binds to site 5, the physiological effect is a prolonged opening of the channel which is caused by a destabilization of the inactivated conformation of the protein (G. Jeglitsch, K. Rein, D.G. Baden and D.J. Adams, 1994, Biophysical Journal, vol. 66, A323). Since the brevetoxins and ciguatoxin bind competitively to the same site of the channel, there must be some structural common denominator that is 'recognized' by the receptor site on the channel protein, that is, a common pharmacophore. Electrophysiological studies of sodium channels show four effects of brevetoxin B (PbTx-2 or PbTx-3): (i) a shift of activation potential to more negative values (channels are open at membrane potentials where they are normally closed) [5]; (ii) population of a subconductance state in addition to the normal conducting state (with slowed kinetics of ion transport) (G. Jeglitsch, et al., as above); (iii) induction of longer channel 'mean open times' ([6] and G.

Jeglitsch, K., et al., as above); and (iv) inhibition of channel inactivation (the inactivated conformation is destabilized or made kinetically inaccessible) [2].

Based on the structure–activity relationships of several brevetoxin B derivatives [7] and molecular modeling of the brevetoxin B [8] and brevetoxin A [9] backbones (Fig. 1), we have recently postulated that the 'common pharmacophore' for the toxins that bind to site 5 is a roughly cigar-shaped molecule, 30 Å long, bound to its receptor primarily with hydrophobic and nonpolar solvation forces, possibly aided by strategically placed hydrogen bond donors near the site of the A-ring carbonyls' [9]. The notion of hydrogen bonding by the A-ring lactone carbonyl was derived from a comparison of the structures of the brevetoxin A and B backbones and also a comparison of the binding of PbTx-3 (brevetoxin B backbone, Fig. 1) with that of 2,3-dihydro PbTx-3. There is a seven-fold loss of binding affinity when the A-ring double bond is reduced, consistent with the expectation that a saturated lactone is less basic than an
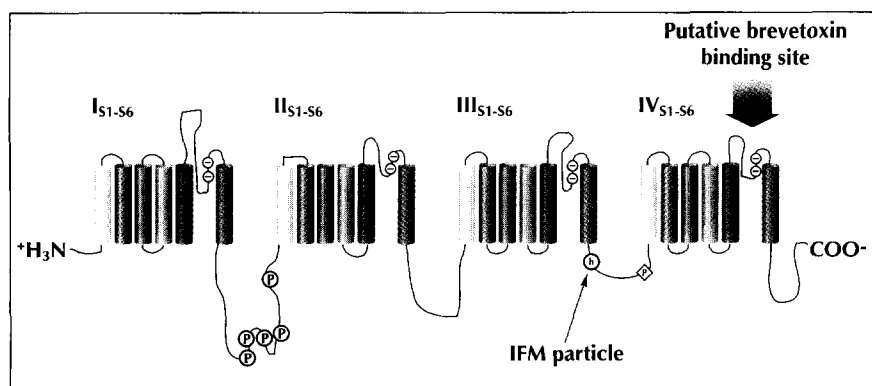

Fig. 2. Cartoon representing the secondary structure of the α-subunit of the voltage-gated sodium channel. The subunit comprises four homologous domains and six subdomains, with intracellular and extracellular loops. The position of the IFM particle, which is thought to be involved in inactivation of the open channel, is shown. Amino-acid residues forming the IFM particle are represented by ⓑ. A protein kinase C phosphorylation site is indicated by ◈.

α,β-unsaturated lactone, making it a poorer hydrogen bond acceptor [7].

To further test the common pharmacophore hypothesis, we have prepared by total synthesis or degradation three brevetoxin derivatives (Fig. 3) for bioasssay. These include a 'truncated' brevetoxin B (compound 1) [10], that has all the functionality of brevetoxin B but is 10 Å shorter, a brevetoxin PbTx-3 derivative lacking the A-ring carbonyl oxygen, (compound 2), and the 2,3-dihydro A-ring-reduced diol, (compound 3), reported previously [7]. We report here the results of the testing of these analogs in synaptosome binding and electrophysiological studies, which provide strong support for the pharmacophore hypothesis stated above. We also offer an extended hypothesis to explain the mechanism for the observed inhibition of sodium channel inactivation induced by brevetoxins.

Results and discussion

Synaptosome binding by brevetoxin and analogs

The binding affinities of the three analogs described above to synaptosomes were determined using a competition assay (Table 1). Three native brevetoxins were also examined. Of the analogs, only the 1-desoxy PbTx-3 displaces tritiated brevetoxin in a competitive fashion, with 50 % inhibition at nanomolar concentrations. The other analogs fail to displace tritiated PbTx-3, even at micromolar concentrations. The loss of the A-ring oxygen (compare PbTx-3, Fig. 1 to 1-desoxy PbTx-3,

Table 1. Synaptosome binding data for native brevetoxins and derivatives.

Compound	IC_{50} (nM)[a]	range
PbTx-1	4.4	2.9–3.4
PbTx-2	15.02	6.36–7.93
PbTx-3	8.48	6.09–11.81
1	>10 000	–
2	36.5	26.8–49.6
3	>25 000[b]	–

[a]Measured against 1.8 nM [³H]-PbTx-3. Values for native toxins differ slightly from those published previously since we now use a different curve-fitting program to process the data. [b]Data taken from [8].

compound 2, Fig. 3) results in a seven-fold loss in binding affinity, which is qualitatively consistent with the presence of a hydrogen bond donor in the vicinity of the A-ring lactone carbonyl, as stated in the pharmacophore hypothesis above. Quantitative interpretation is not possible, since the differences between a methylene and a carbonyl group also include differences in steric requirements, electrostatics, and dipoles; however, interesting differences in the electrophysiological effects of the compounds are observed and are discussed below.

Particularly intriguing is the inability to displace tritiated PbTx-3 with the shorter analog, truncated PbTx-2 (compound 1), which has a very similar structure, including all
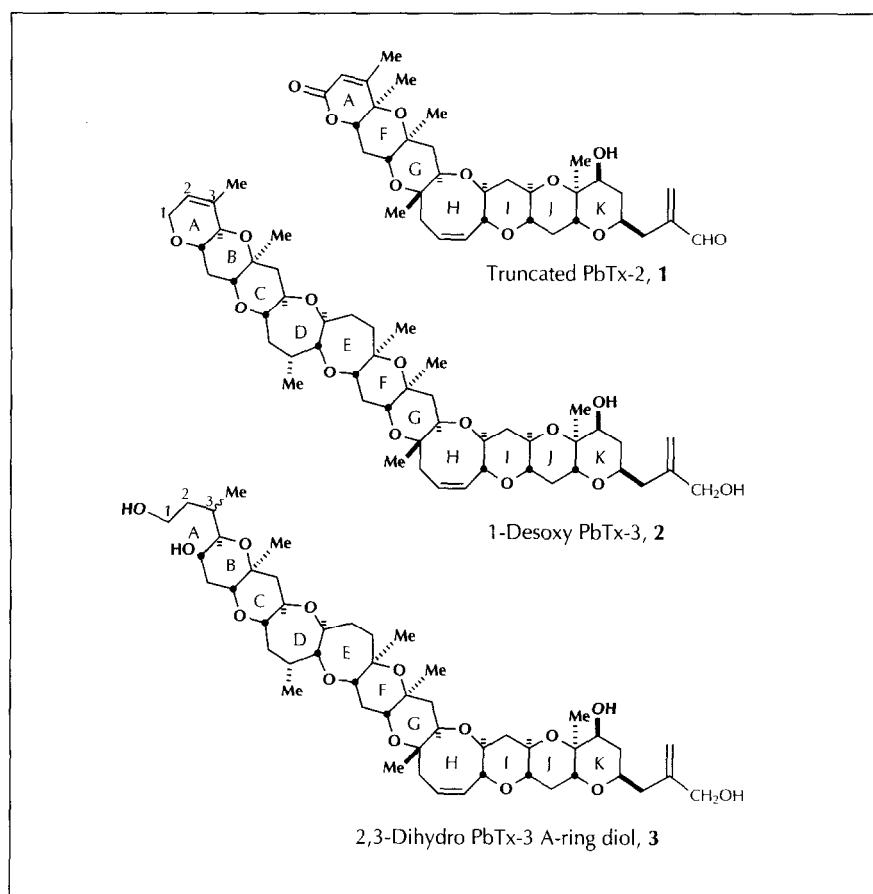

Truncated PbTx-2, **1**

1-Desoxy PbTx-3, **2**

2,3-Dihydro PbTx-3 A-ring diol, **3**

Fig. 3. Brevetoxin B derivatives used in this study.

the functional groups of PbTx-2, the same absolute configuration, and very similar hydrophobicity. We also find that the truncated PbTx-2 is not toxic to mosquito fish (*Gambusia affinis*) at μM concentrations, whereas PbTx-2 has an LC_{50} of 14 nM [7]. When compared to the global minimum conformation of PbTx-2 [8,9], compound **1** is 10 Å shorter. Figure 4 shows a Corey–Pauling–Koltun (CPK) model of the PbTx-2 backbone in its lowest energy conformation and a CPK model of the truncated backbone of compound **1**. Figure 5 shows displacement curves from the synaptosome binding assay for compounds **1** and **2**, with PbTx-3 included for comparison. (PbTx-2 and PbTx-3 have different side chains at the tail end of the molecule, but we have already shown that these differences have a negligible effect on binding affinity [7]).

Examination of the two CPK models in Figure 4 does not reveal any obvious structural features that could account for the dramatic difference in the binding affinity of PbTx-3 and compound **2**. Truncated PbTx-2 differs from PbTx-2 in two ways however: the overall length of the molecule is reduced, and the flexibility normally endowed on the molecule by the two seven-membered rings (D and E of PbTx-2, Fig. 1) is missing. Molecular modeling studies [8,9] have shown that the eight-membered H-ring of the brevetoxin B backbone is locked into a boat–chair conformation by the torsional constraint of the double bond. Thus, the eight-membered rings of both PbTx-2 and the truncated analog **1** are rigid. Reduction of this double bond changes the global minimum to a crown conformation, resulting in a ~1000-fold loss in binding affinity [7]. Because all the six-membered rings of the brevetoxin B backbone are rigid by virtue of their *trans*-decalin-like nature, the only conformational motion available is at the seven-membered D and E rings. This structural feature is removed in the truncated analog **1** (Fig. 3).

A comparison of the low-energy minima of both the brevetoxin A and B backbones, and consideration of the fact that both ligands bind competitively to the same receptor, led us to conclude that the conformation of
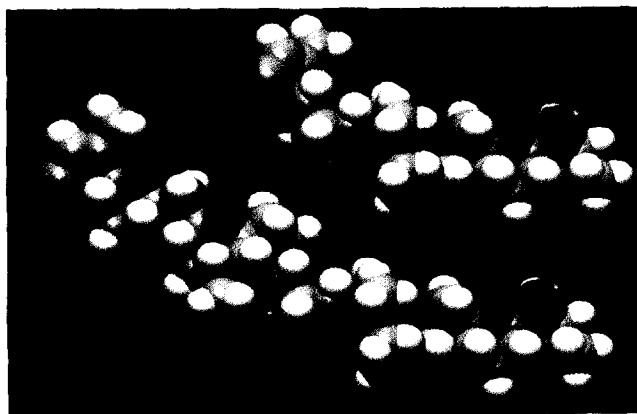

Fig. 4. Computer-rendered CPK models of truncated PbTx-2 (compound **1**) (top) and PbTx-2 (bottom), oriented with the 'head' of the toxin (see Fig. 1) to the left. The side chain at the 'tail' of the molecules has been replaced by a methyl group.
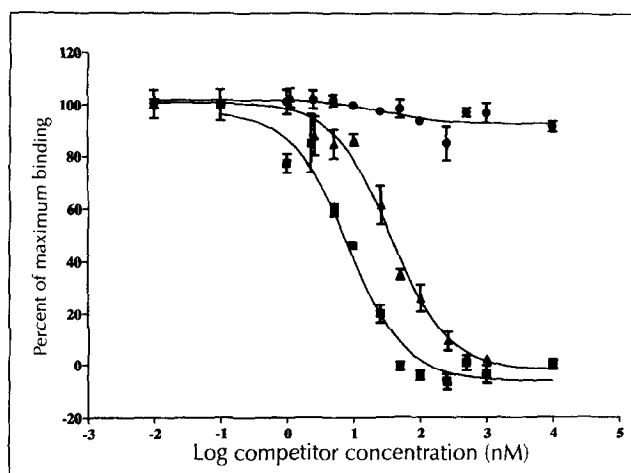

Fig. 5. Compound **1** binds to receptor less well than PbTx-3 or compound **2**. Displacement of 1.8 nM tritiated PbTx-3 with unlabeled PbTx-3 (■), unlabeled compound **1** (●) or unlabeled compound **2** (▲) is shown as curves of percent of maximum binding versus log of competitor concentration.

brevetoxin B, when bound to site 5, is straight (as opposed to some possible conformations that have a significant bend at the seven-membered D/E rings) [9]. Truncated PbTx-2 has a shape that is similar to the lowest energy linear conformation of the brevetoxin B backbone (Fig. 4). If our conclusions about the conformation of brevetoxins bound to site 5 are correct, then the only difference that can account for lack of binding is the length of the toxin.

Electrophysiological effects of brevetoxin and analogs

We next determined the effects of PbTx-3 and truncated PbTx-2 (compound **1**) on depolarization-activated single-sodium-channel currents (Fig. 6). There are two structural differences between these molecules: the truncation and the K-ring side chain (PbTx-2 is an aldehyde and PbTx-3 is an alcohol). However, at concentrations of 30–500 nM, PbTx-2 and PbTx-3 are indistinguishable electrophysiologically (data not shown). PbTx-3 clearly has profound effects on channel opening at 30 and 500 nM concentrations, whereas truncated PbTx-2 (**1**) shows very little effect even at 1 μM concentration.

The mean open times of single sodium channels in the absence (control) or presence of 30 nM PbTx-3 or 10 μM truncated PbTx-2 are shown in Fig. 7a. In the absence of added ligand (control), the channel activates at −40 mV, whereas in the presence of either PbTx-3 or truncated PbTx-2, the channel activates at −50 mV (i.e., both compounds produce a negative shift of 10 mV in the activation potential). Channel openings are also observed at the resting membrane potential (−50 mV). The mean channel open times in the presence of 30 nM PbTx-3 are increased significantly compared to control conditions. The mean channel open times in the presence of truncated PbTx-2 (**1**), at similar concentrations, are nearly the same as the control. Even at 10 μM concentrations (a 333-fold increase), the effect of truncated PbTx-2 is small at potentials more negative than −30 mV. Figure 7b shows the effect of ligand concentration on mean open times at

Fig. 6. Depolarization-activated single sodium channel currents in the absence (control) or presence of **(a)** 30 and 500 nM PbTx-3, and **(b)** 1 and 10 μM truncated PbTx-2 (compound 1). Typical consecutive traces (100 ms) of unitary sodium currents (downward deflections) obtained upon depolarization to –40 mV and –10 mV from –100 mV in (a), and to –30 mV from –100 mV in (b). Measured resting membrane potential (RMP) ≈ –50 mV. The voltage protocol is shown above. Pipette and bath solutions are described in Materials and methods.
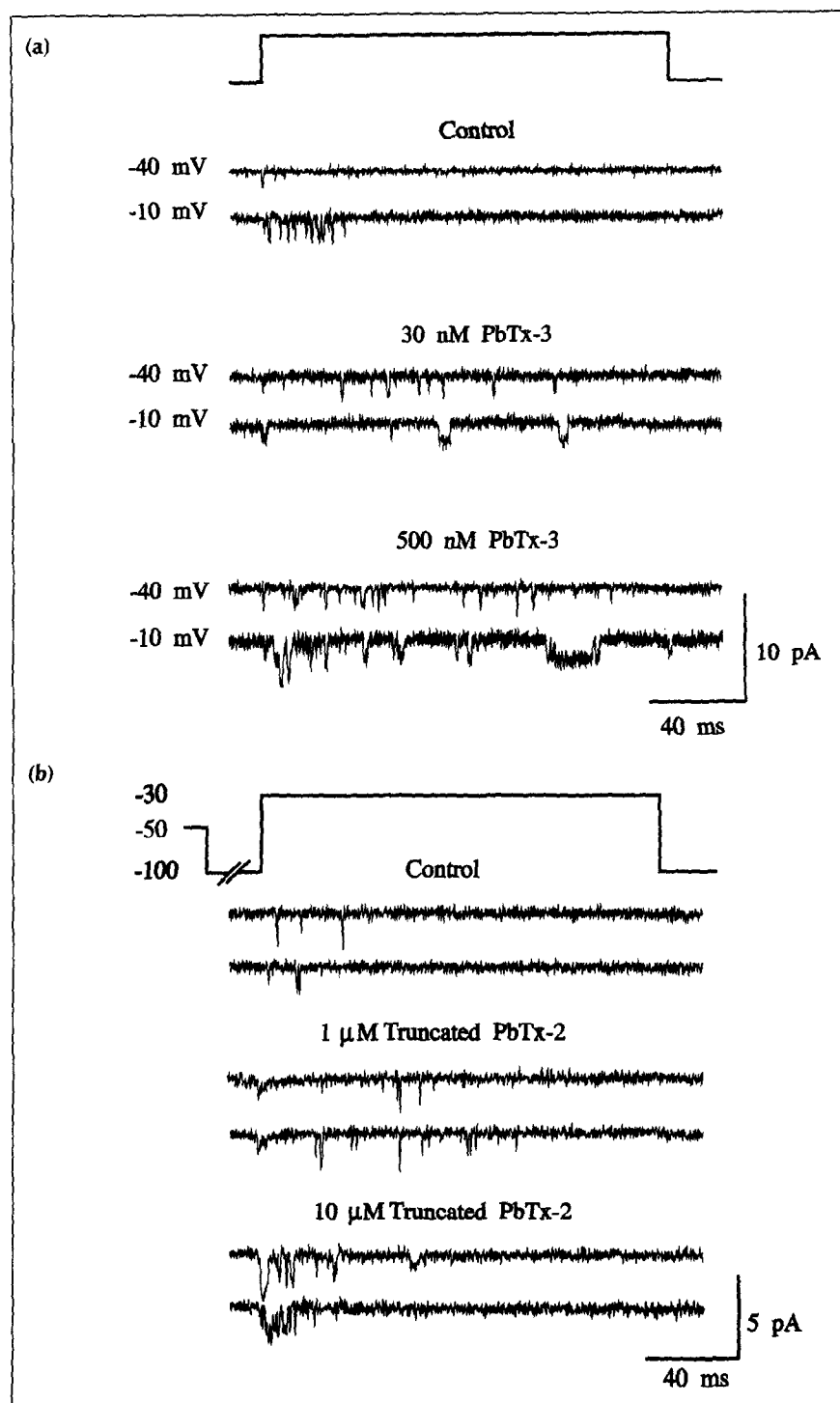
selected potentials. Here again, note that the truncated toxin has negligible effect compared to PbTx-3 at potentials from –40 to 0 mV. Truncated PbTx-2 is not ichthyotoxic; of the four characteristics of native brevetoxins mentioned above, it does not possess the binding affinity of a native toxin, and it does not prolong mean open times or inhibit inactivation, although it does shift the activation potential. It may therefore be considered to be a partial brevetoxin agonist.

Steady-state recordings of channel openings in the presence of 500 nM PbTx-3 and 1 μM 1-desoxy PbTx-3 (compound 2) are shown in Figure 8. Under control

conditions, the channel briefly opens following depolarization, then becomes inactivated. If the depolarizing potential is maintained, the channel will remain in the inactivated conformation until the membrane is hyperpolarized, at which point the conformation moves from the inactivated to the closed state. In the presence of 500 nM PbTx-3 (Fig. 8a), channels open and tend to remain open. In the presence of 1-desoxy PbTx-3 at twice the concentration (1 μM, Fig. 8b), the channels exhibit only brief open times, but repeatedly open (and close) over the entire sampling time (unmodified channels open only once). This observation is all the more surprising since the only difference between the two ligands is
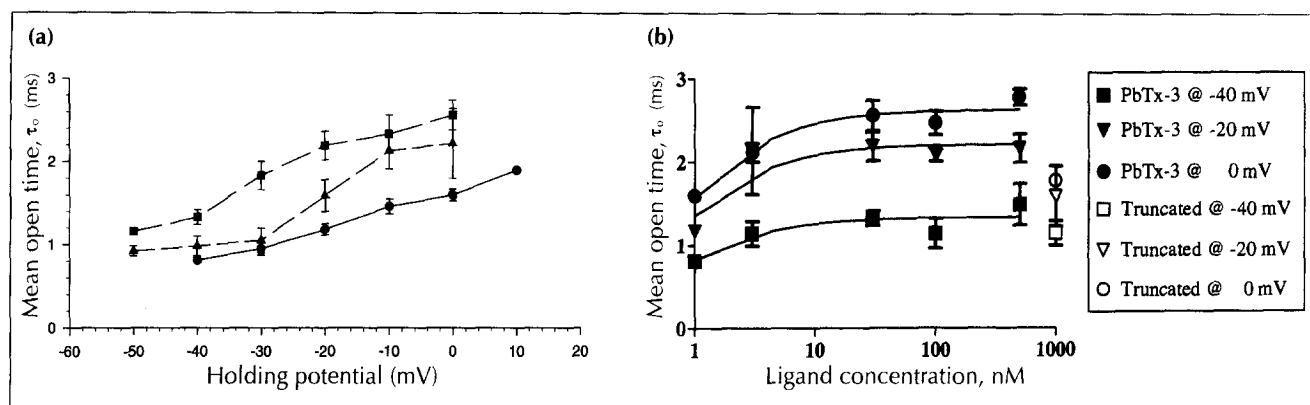
(a)

(b)

■ PbTx-3 @ -40 mV
▼ PbTx-3 @ -20 mV
● PbTx-3 @   0 mV
□ Truncated @ -40 mV
▽ Truncated @ -20 mV
○ Truncated @   0 mV

Fig. 7. Effects of PbTx-3 and truncated PbTx-2 on channel opening. **(a)** The mean channel open time (τ_o) obtained at different membrane potentials in the absence of ligand (●) or presence of 30 nM PbTx-3 (■) or 10 μM truncated PbTx-2 (▲). Each data point represents mean ± one standard error of the mean τ_o calculated from at least three experiments. **(b)** The mean channel open time as a function of ligand concentration for PbTx-3 and compound **1** ('truncated') at –40, –20, and 0 mV membrane potential.

the presence or absence of the carbonyl oxygen in the A-ring lactone. Regarding the four characteristics of native brevetoxins mentioned above, 1-desoxy PbTx-3 (compound **2**) clearly does not induce longer mean open times, although it does shift the activation potential and inhibit inactivation. Compound **2** therefore may also be considered to be a partial brevetoxin agonist.

The A-ring diol (compound **3**) [7], under steady-state conditions at 10 μM concentration, induces the channels to populate five subconductance states (Fig. 9). Subconductance states are, by definition, ion-conducting conformations of the channel in which the rate of sodium ion transport differs from the 21 pS fully open channel. A conductance substate arises whenever a channel occupies a
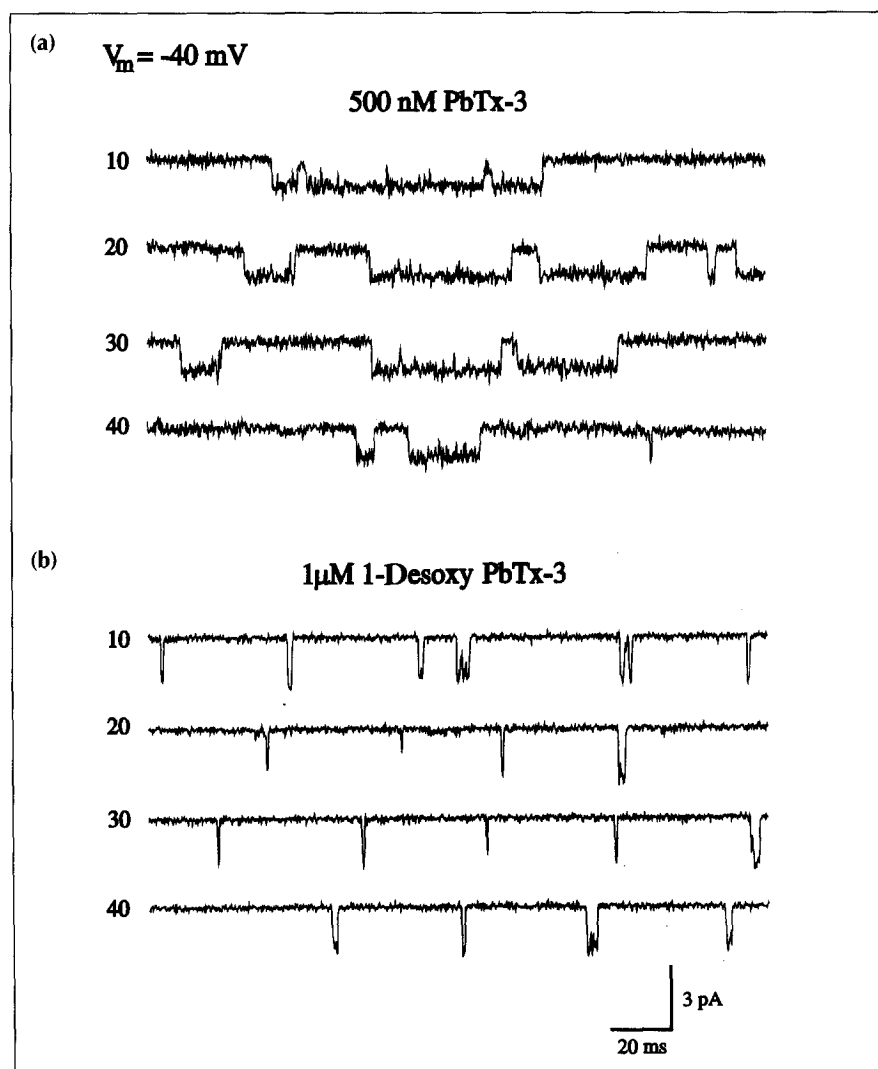

(a) $V_m = -40$ mV

500 nM PbTx-3

10
20
30
40

(b)   **1μM 1-Desoxy PbTx-3**

10
20
30
40

3 pA

20 ms

Fig. 8. Steady-state recordings of single sodium channel openings in the presence of **(a)** 500 nM PbTx-3 or **(b)** 1 μM compound **2**. The membrane potential was held constant at –40 mV over a period of 1 min. 200 ms current traces, obtained after 10, 20, 30, and 40 s are shown for each compound.

Fig. 9. Steady-state recordings of single sodium channel openings in the presence of 10 µM compound **3**. The membrane potential was held at –40 mV. Examples of different subconductance levels (I–V) and the closed state (C) are shown.
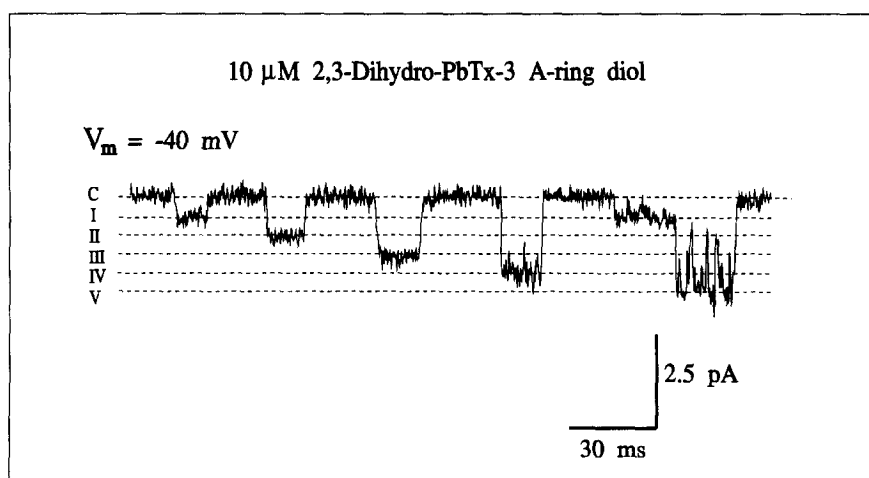
conformational state with an energy profile for ion permeation that is different from the energy profile for the main conductance state. Under control conditions, during single-channel recordings, most openings are to the main conductance state (V), but subconductances are seen when the channel visits other less favorable conformations [11]. In the case of toxin interaction with channels, the toxin stabilizes these less favorable channel conformations, allowing the subconductance states to be observed.

In summary, experiments that measure specific binding to synaptosomes and single sodium channel currents indicate a substantial loss of binding and significantly modified activity when the brevetoxin B backbone is shortened by 10 Å (truncated PbTx-2 (**1**)). Removal of the A-ring carbonyl oxygen (desoxy PbTx-3 (**2**)) results in a seven-fold decrease in binding and a loss of the ability to prolong the channel open time. Reduction of the A-ring to a saturated diol induces a significant loss of binding affinity [7], while affording a ligand capable of populating five channel subconductance states.

These observations are consistent with the common pharmacophore hypothesis stated above [9]. We now wish to extend this hypothesis to include a model of how the toxin binds to the channel and affects conformational motion (Fig. 10). Because the truncated toxin (compound **1**) shows a low binding affinity and does not alter sodium channel kinetics, but still produces a shift of the activation potential, we conclude that the 'cigar shape' may be all that is necessary to induce this last effect. Furthermore, we know from photoaffinity studies that the 'tail' of the brevetoxin B backbone resides in the vicinity of the S5–S6 extracellular loop of domain IV of the protein (see Fig. 2) [12,13]. Based on the fact that compounds in which the A-ring is modified show altered kinetics of inactivation and population of subconductance states, we believe that the 'head' of the toxin is in the vicinity of the IFM particle (III_{S6}–IV_{S1} intracellular loop, see Fig. 2) on the cytoplasmic side of the membrane.

It remains to be determined precisely where the brevetoxin molecule lies with respect to the various helices
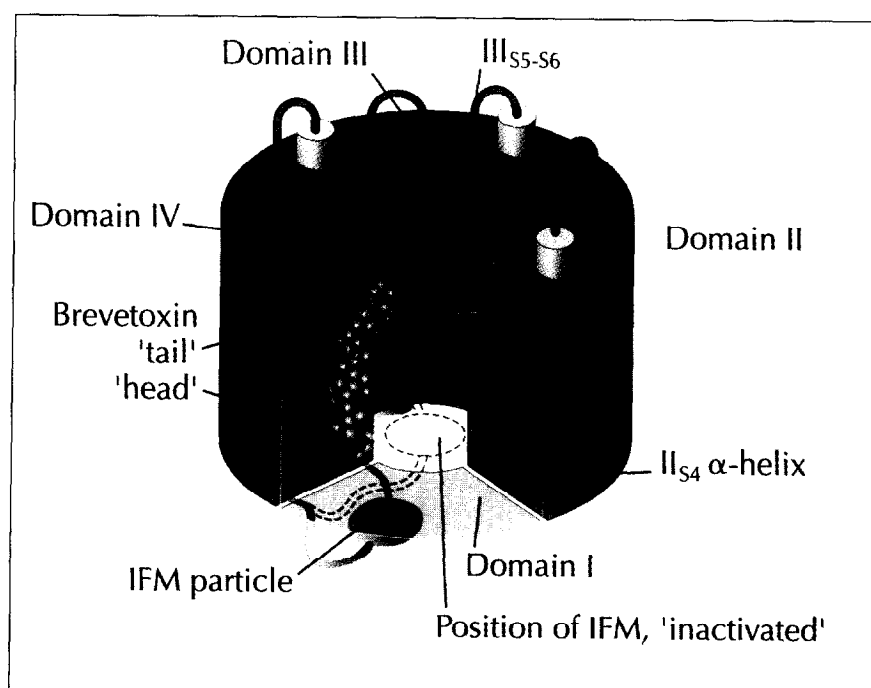

Fig. 10. Hypothetical model of the binding of brevetoxin to site 5 of the voltage-gated sodium channel, with the tail of the model near the I_{S5-S6} loop and the head near the inactivation particle. The cartoon of the channel protein is based on that in [4].

and domains of the voltage-sensitive sodium channel, but from these results in the context of earlier work it is clear that the orientation is 'head down' near the interface of domains I and IV of the channel.

Significance

Brevetoxins are neurotoxins that pose a significant health risk because of their neurotoxic effects, as manifested in neurotoxic shellfish poisoning. Brevetoxins and ciguatoxin bind in a 1:1 stoichiometry to a unique site on the channel protein α subunit known as site 5, with IC_{50} values in the nanomolar range. The common pharmacophore of the brevetoxins is thought to be a 30-Å long cigar-shaped molecule that binds to the protein primarily by hydrophobic interactions, but hydrogen bonding to the A-ring carbonyl seems to be important as well.

The voltage-gated sodium channel opens following depolarization; the open channel is thought to be inactivated by movement of a segment of one of the intracellular loops, the IFM particle, to block the channel. We propose a model in which the toxin binds to an open (conducting) conformation of the channel protein and the head of the toxin physically obstructs the normal motion of the IFM particle, directly or indirectly (much as a pencil might be used to stop a door from closing). This model accounts for all the known facts regarding brevetoxin toxicity, particularly the kinetic inaccessibility of the 'inactivated' conformations. Finally, the C-1 carbonyl oxygen is now explicitly implicated in the prolonging of 'mean open times', probably through hydrogen bonding.

The structure-activity relationships derived from the analogs studied here indicate clearly that the four properties of the brevetoxins are not produced by a single structural feature, since they can be 'decoupled' in modified ligands. This implies that brevetoxin derivatives can be produced which are partial sodium channel agonists, and further that it is distinctly possible that brevetoxin antagonists could be developed.

Materials and methods

Synthesis

Details for the synthesis of truncated PbTx-2, (1) [10], and diol **3** [7] have been published. 1-Desoxy PbTx-3, compound **2**, was prepared in two (unoptimized) steps from PbTx-3: 1) sodium borohydride reduction to the A-ring diol having the 2,3-double bond intact, as described previously [7]; 2) Mitsunobu cyclization [14,15]: the A-ring diol (6.0 mg, 6.67 x 10^{-3} mmol) was dissolved in 10 ml ether (distilled from sodium benzophenone). To this solution was added Ph_3P (5.35 mg, 2.04 x 10^{-2} mmol), followed by diethylazodicarboxylate in ether (10 μl, 181 mg ml^{-1}). After stirring at room

temperature for 4 days, the reaction mixture was partitioned between ether and water. The organic phase was condensed, then taken up in 2 ml MeOH and purified by reversed phase high-pressure liquid chromatography (HPLC) (C_{18} column, 85 % aq. MeOH eluent). Concentration of the eluate afforded 0.740 mg (12 %) of compound **2**. High-resolution mass spectrometry (fast atom bombardment, nitrobenzyl alcohol (NBA) matrix) MH^+ calc'd. for $C_{50}H_{75}O_{13}$ 883.5207, found 883.5198. The ^1H NMR (400 MHz) showed the following diagnostic peaks: δ5.35 (1H, bs, C-2 vinyl proton), 4.25–4.16 (2H, broadened AB quartet, buried under other allylic ether signals).

Synaptosome binding

Synaptosomes were prepared by the method of Dodd *et al.* [16] from male Sprague–Dawley rats (200–250 g). Total binding was measured using a rapid centrifugation technique described previously [17]. Non-specific binding was measured in identical incubation tubes by adding 10 μM unlabeled PbTx-3 to completely displace all specifically bound radioactive toxin (radioactivity that cannot be displaced by excess ligand is non-specific [18]). All binding experiments were performed in standard binding medium (SBM) consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg ml^{-1} bovine serum albumin, and 0.01 % Emulphor EL-620, a non-ionic detergent used as an emulsifier. The latter was required to solubilize the high concentration of competitor toxins. A suspension of synaptosomes (40–80 μg total protein in 100 μl SBM) was added to microfuge tubes containing [^3H]PbTx-3 (1.78 nM) and competitor (12 different concentrations in the range 0.1 nM–10 μM) for a total final volume of 1.0 ml. After mixing and incubating at 4 °C for 1 h, the tubes were centrifuged for 3 min at 15 000 x g. Supernatant solutions were aspirated and the remaining pellet rapidly rinsed with 0.5 ml of cold (0 °C) SBM. The pellets were transferred to liquid scintillation vials and bound radioactivity was counted on a Beckman LS 1801 liquid scintillation counter in 3.0 ml of Ecolume (ICN Biomedicals, Costa Mesa, CA). IC_{50} data were obtained by fitting a one site competition curve to displacement data using the program Prism (v 1.0, GraphPad, Inc.). Binding was determined to be competitive using the method of Cheng and Prusoff [19].

Electrophysiology: I. Cell preparation

The mammalian nodose ganglion is a visceral afferent sensory ganglion which is located along the vagus nerve at the level of the bifurcation of the carotid arteries. The nodose ganglia were dissected from newborn rats (1–2 weeks old), killed by intraperitoneal injection of sodium pentobarbital. The ganglia were removed, transferred into Krebs–Ringer solution and cleaned of connective tissue. The isolated ganglia were pinned out and digested with collagenase (0.6 mg ml^{-1}, Wothington Type) and protease (0.4 mg ml^{-1}, Sigma). After 1 h at 37 °C the ganglia were transferred to a sterile culture medium (Dulbecco's modified Eagle medium, 10 % (v/v) fetal calf serum, 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin) and also mechanically dissociated using forceps. The tissue fragments were triturated with a sterile fine-bore Pasteur pipette. The dissociated neurons were plated onto laminin-coated 18 mm glass coverslips, placed in a 35 x 10 mm plastic culture dish (Falcon) and incubated at 37 °C in a 95 % air, 5 % CO_2 atmosphere. The neurons were placed in tissue culture 48–72 h prior to use. All neurons had a diameter between 15 μm and 25 μm.

II. Solutions

The composition of the physiological salt solution was (mM): 140 NaCl, 3 KCl, 0.6 MgCl$_2$, 2.5 CaCl$_2$, 7.7 glucose, 10 histidine, pH = 7.4. The extracellular solution for single-channel recordings contained (mM): 140 NaCl, 3 KCl, 1 CaCl$_2$, 0.6 MgCl$_2$ 7.7 glucose, 10 HEPES, adjusted to pH 7.4 with NaOH. The pipette solution for single-channel recordings contained (mM) 140 NaCl, 3 KCl, 0.6 MgCl$_2$, 2.5 BaCl$_2$, 30 tetraethylammonium chloride (TEACl), 7.7 glucose, 0.1 4,4'-diiothiocyanato-2,2'-stilbene disulfonic acid salt (DIDS), 10 HEPES, and was adjusted to pH 7.4 with NaOH. For single-channel recordings in the presence of toxin, either tetrodotoxin (TTX), PbTx-3, or one of the derivatives was added to the patch pipette solution at the concentrations stated. TTX was dissolved in distilled water, brevetoxins and analogs in absolute EtOH. Experiments were conducted at room temperature (22–23 °C).

III. Electrophysiological recording

Single-sodium-channel currents were recorded from cell-attached membrane patches using the patch clamp technique [20]. Patch pipettes were pulled from thick-walled borosilicate glass (GC150F, Clark Electromedical Inst., UK). The tip was fire-polished on a microforge and coated with Sylgard (Dow Corning) to reduce electrode capacitance. Pipettes had tip resistances of 10–15 MΩ when filled with pipette solution. Patch electrodes were sealed against the membrane by suction, leading to seal resistances between 5–10 GΩ. Voltage steps were applied to the patch pipette with pulse protocols generated by a computer (PC 486D/50 MHz) and pClamp programs (Axon Instruments, Inc., CA). The membrane 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 using an Axopatch 200A patch clamp amplifier (Axon Instruments), 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 using pClamp programs. Linear leak and capacitative currents were subtracted from current traces using records which contained no openings during the depolarizing (200 ms) pulse. Steady-state single-channel currents were recorded on videotape using an A/D recorder adapter (PCM-2, Medical Systems, NY) prior to computer analysis. All data were analyzed by using pClamp version 5.5.1 and Filecut version 2.1. Numerical data are represented as the mean ± one standard error of the mean.

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